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Polyethylenimine: A very useful ionic polymer in the design of immobilized enzyme biocatalysts

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Abstract

This review discusses the possible roles of polyethylenimine (PEI) in the design of improved immobilized biocatalysts from diverse perspectives. This includes their use to activate supports and immobilize enzymes via ion exchange, as well as to improve immobilized enzymes by coating with PEI. PEI is a polymer containing primary, secondary and tertiary amino groups, having a strong anion exchange capacity under a broad range of conditions, and the capability to chemically react with different moieties on either enzyme or support. Also, as a multifunctional polymer, it has been modified stepwise to introduce different functionalities in the same polymer. This polymer (in combination with other anionic ones) permits the generation of "saline" environments around enzyme molecules, improving enzyme stability in the presence of hydrophobic compounds. The use of PEI as a physical glue useful to crosslink enzymes subunits in multimeric enzymes, monomeric enzymes immobilized via physical interactions or production of enzyme multilayers will be specially emphasized as new open avenues for enzyme coimmobilization. The coimmobilization of enzymes and cofactors using PEI may become one of the future developments allowed through an adequate use of this polymer and new pathways towards the design of enzyme combi-catalysts for their use in cascade reactions. Some unexplored but suggested uses derived from the properties of PEI are also proposed in the review, like the use of the buffering power of this multifunctional polymer to avoid pH gradients inside the biocatalyst particle. Thus, although PEI has been a largely popular polymer in biocatalysts design, it looks that a long and in some cases almost unexplored road lies ahead.

Key words: generation of nanoenvironments, physical crosslinking, multimeric enzymes, coimmobilization of enzymes and cofactors, CLEAs crosslinking, cascade reactions

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1. Introduction: The role of immobilization and physicochemical modification of enzymes in the design of industrial biocatalysts

One of the main goals of chemistry in the 21st century is the design of greener and more environmentally sustainable processes.[1–3] Enzyme biocatalysis has risen as a very suitable pathway towards this goal;[4] enzyme catalysis is selective, specific and may be performed under mild conditions.[5–8] However, enzymes have evolved to fulfill the natural requirements of microorganisms, e.g. capacity to respond to changes in the medium or adaptation to stress conditions, and they have many properties that greatly differ from those required from an industrial catalyst.[9–11] For example, enzymes are moderately stable, and may be inhibited by products, substrates or even non-related compounds.[12] One additional problem is that most proteins are water soluble, making recovery of this expensive catalyst complex (almost impossible in some cases). These problems have hampered their industrial implementation. Nowadays, revolutions in many areas related to the design of biocatalysts, such as metagenomics,[13,14] directed evolution,[15,16] or site-directed mutagenesis,[17] have reduced the price of enzymes and have thus increased their availability. In this review, we will focus on enzyme immobilization. Enzyme immobilization needs to be performed to facilitate enzyme recovery (Figure 1). This prevents product contamination and favors enzyme reuse, provided that the immobilized enzyme is stable enough to be reused.[18–20]

The necessity for enzyme immobilization has spurred an intensive research to couple immobilization to the improvement of other enzyme features. It has been shown that a proper immobilization may improve enzyme stability (e.g., via multipoint or multisubunit immobilization (Figures 2 and 3), generation of suitable enzyme nano-environments (Figure 4)), but may also improve enzyme activity (by generating more active conformations or using internal substrates or products

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gradients), specificity or selectivity.[9,21–26] Even enzyme purity may be enhanced using a properly designed immobilization protocol, saving time, avoiding side reactions that can decrease process yields or selectivity/specificity, and decreasing the amount of support required to immobilize a fixed amount of the target enzyme.[27] All these enzyme features are related in a certain sense. For example, as stated by J. Woodley,[28] from a practical point of view, enzyme stability should be considered not just as an operational lifetime indicator, but should be measured as the amount of synthesized product per biocatalyst mass (price of product/price of used biocatalyst). This means that if immobilization of the enzyme increases the operational half-life, and also reduces the reaction time course (e.g. due to a reduction of inhibition problems or an improved enzyme activity), the "stability" of the enzyme will be boosted by all these improvements.

However, enzyme immobilization has some costs that must be compensated for by the gains: support and processing costs. While much attention has been paid to the support costs (even though it is much cheaper than the enzyme), it should be considered that if a support that has a higher cost (let us suppose by a 10-fold factor) permits a higher loading, a higher enzyme stability and productivity, its price may become irrelevant. Moreover, reversible enzyme immobilization methods have the advantage of reducing the weight of the support price in the immobilized enzyme biocatalysts, because the support may be reused after enzyme inactivation: the inactivated enzyme may be released and fresh enzyme may be added (Figure 1).[23] The main point is to have so strong an enzyme reversible immobilization that the enzyme cannot become desorbed during operation. Physical adsorption is the simplest reversible immobilization,[29] but it is not devoid problems because the support surface will always be physically active and that can generate some problems regarding enzyme support uncontrolled interactions.[30] Furthermore it is not possible to design approaches for protein reactivation using unfolding/refolding strategies.[23] However, in certain cases, physical adsorption of the enzyme may produce some enzyme stabilization, like in the case of multimeric enzymes, if this immobilization

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involves all enzyme subunits and prevents enzyme dissociation (Figures 2 and 3).[24] Interfacial activation of lipases on hydrophobic supports[31] is perhaps one of the most successful examples where physical adsorption may stabilize biocatalysts even to a larger degree than multipoint covalent attachment[32,33] due to the existence of a specific enzyme form in these supports that is more stable than the lipase in equilibrium conformation.[34] However, even in this case, the enzyme may be desorbed during operation and reduce the application of this method.[35,36]

Physical or chemical modification of enzymes is another way to improve enzyme properties (Figure 5).[37,38] The introduction of intramolecular crosslinkings with small bifunctional reagents may greatly increase enzyme rigidity,[39,40] while inter-subunit crosslinking of multimeric enzymes may avoid subunit dissociation (Figures 2 and 3).[24] There are many examples where the enzyme properties (activity, stability, specificity) have been improved by a global modification of the enzyme surface (Figure 5).[41–44] Immobilization and physicochemical modification of enzymes are not unrelated tools to improve enzyme properties, but may be used in a coupled way to improve the final performance of the enzyme biocatalyst (Figure 5).[45,46] In many instances, immobilization of the enzyme may facilitate further modifications (chemical or physical). In other cases, chemical modification is designed to improve enzyme immobilization (Figure 3).[47]

In this review, the use of polyethylenimine as a reagent for the preparation of immobilized enzyme biocatalysts will be reviewed, both as a method to activate supports as ion exchangers or to physically modify enzymes before or after their immobilization. This multifunctional polymer has been employed in biocatalysis design in many instances and with very different objectives. The properties of this polymer and some of their applications will be presented and discussed, e.g., stabilization of multimeric enzymes (Figures 2 and 3), stabilization of enzymes versus organic solvents or oxygen (Figure 6), immobilization of enzyme multilayers (e.g., coimmobilization of enzymes) (Figure 7) or coimmobilization of enzymes and cofactors. The potential of this polymer in biocatalyst

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design is already quite impressive and considering the latest applications, it is safe to assume that it has not been fully exploited yet.

2. Production and features of PEI

Polyethylenimine (PEI) is one of the most widely used synthetic polycations in various applications because of its chemical functionality arising from the presence of cationic primary (25%), secondary (50%), and tertiary amines (25%).[48,49] PEI is formed by the linking of iminoethylene units and can have linear, branched, comb, network, and dendrimer architectures depending upon its synthesis and modification methods, which greatly influences its properties, both physical and chemical.[50] Furthermore, these synthetic approaches enable PEI to be available in a wide range of molecular weights. At room temperature, branched PEI (BPEI) is a highly viscous liquid while linear PEI (LPEI) is a solid. PEI has several attractive features for its use in widespread applications, such as low toxicity, ease in its separation and recycling, and (last but not least) its being odorless. In addition to these attractive features, there is a distinct feature of PEI which places it ahead of other polyions (e.g. polyallilamine or chitosan) when it comes to loading, and which justifies its widespread use in fields as varied as detergents, adhesives, water treatment, cosmetics, carbon dioxide capture,[51–54] as DNA transfection agent, and in drug delivery:[55–58] despite being a weak polymeric base with pK_a values between 7.9 and 9.6, it possesses a high ionic charge density, which in practical terms translates in being a more cost-effective material. This derives in either the possibility of reaching the same loadings with reduced amounts of the polymer (which would colloquially mean "getting a bigger bang for the buck") or reaching loadings which are beyond the reach of the aforementioned examples while avoiding enzyme agglomeration thanks to its multibranched network.

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Commercial polyethylenimine usually has a branched structure. BPEI is soluble in polar solvents as water, ethanol, and acetone and depending on the molecular weight is partially soluble in benzene and tetrahydrofuran [59,60]. This molecule will be the most interesting in biocatalysts design, with a random structure and a capability of covering the surface of supports or proteins. The standard synthetic route for obtaining BPEI involves the electrophilic (or cationic) ring-opening polymerization of unsubstituted ethylenimine with a protonic catalyst such as Lewis acids and their salts, strong Brønsted acids, alkyl halides, strong acid esters, phenols, carboxylic acids, or halogens (Scheme 1).[60] This polymerization causes the formation of a secondary amino group in the polymer chain which is more basic than the monomer. Therefore, chain transfer to the polymer takes place extensively, which ultimately brings about branching. The branched structure from ethylenimine polymerization causes the spheroidal shaped of the BPEI molecules.

Another route for obtaining BPEI contemplates the ring-opening isomerization polymerization of substituted 2-oxazolines with Lewis acids, protonic acids, sulfonate esters, or sulfonic anhydrides as inductors the polymerization (Scheme 2). The reaction rate and yield depend on the substituent, the nature of X^-, solvent, and temperature.[49,60]

On the other hand, linear polyethylenimine is highly crystalline due to its linear structure. Through X-ray studies it was determined that LPEI forms double-stranded helical chains stabilized by interchain hydrogen bonds. The polymer is remarkably hygroscopic, which is due to a transformation from the double stranded helix to a fully extended form upon water adsorption of water. The production of LPEI does not proceed through the ring-opening route of ethylenimine (aziridine) because very high reaction times are required and the yields of the obtained product are very low. Thence, the preparation of LPEI is carried out via ring-opening polymerization (ROP) of 2-ethyl-2-oxazoline followed by hydrolysis, where it is possible to obtain 100% conversion under appropriate conditions (Scheme 3).[48,60]

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3. Use of PEI to immobilize enzymes and proteins on pre-existing supports

Activation of supports by coating their surface with PEI enables enzyme immobilization by anion exchange. This specific use of PEI was reviewed in 1991,[61] but many advances have been made since then. This immobilization protocol requires the possibility of forming several ion bonds between the enzyme and the support, shifting the counter ions that both surfaces will have.[62]

Immobilization of biomacromolecules using these supports having polymeric beds presents some advantages compared to standard anion exchangers.[61,63] Firstly, enzyme adsorption is quite strong, as PEI has multiple cation groups at different distances that may be adapted to the distance between the enzyme groups. Secondly, the polymer is random coil and will not force the enzyme to become distorted when interacting with it via multiple points. Third, the polymeric bed formed by PEI offers a three-dimensional adsorption versus the two-dimensional adsorption of standard supports,[64] enabling the immobilization of more than 80% of the proteins contained in a crude protein extract.[65] However, immobilization remains reversible and the support may be reused (Figure 1).

Considering that supports coated with anionic polymers may also immobilize over 80 % of the proteins under the same conditions,[66] it seems evident that the net charge of the protein is not the reason for enzyme immobilization, but the possibility of establishing many ionic bridges. This idea has permitted the development of different tailor-made ion exchangers for selective immobilization of different proteins.[67]

However, the use of PEI may also raise some problems, e.g., the polymer can interact with internal pockets of the protein (e.g., where some critical cations may be located) leading to enzyme inactivation: this does not occur using small cationic groups on a flat surface. Moreover, the mobility of PEI may give a certain destabilization of the enzyme that is interacting with it as a result.[63] Ion

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exchange should not provide any significant structure rigidification of the enzyme, and even less using PEI due to its high mobility, although some stabilizing effects may be found which are not based in enzyme rigidification, as described in other points of this review.

To maximize the reactive bed volume, the way the coating is produced is critical. Conditions that favor the attachment of many PEI molecules will be preferred versus conditions that favor the spreading of the PEI on the support surface (Figure 8).[65] It is possible to allow the enzyme molecule to penetrate deeper into the polymeric bed, using conditions where the adsorption is not favored and where a higher number of enzyme-support ion bridges is required to fix the enzyme to the support,[68] increasing the adsorption strength and yielding other beneficial effects of immobilization in this kind of supports.[61]

This PEI coating has been used in many different supports and applications. Now, we will review some of the main examples.

3.1. Use of PEI to coat pre-existing macroporous supports to immobilize proteins

3.1.1. Use of supports just activated PEI

Different supports have been activated for a long time with PEI. Using this strategy, it has been found that PEI can have some positive effects on enzyme stability by generating a partition of hydrophobic compounds (like organic solvents or gases like oxygen) from the enzyme environment (Figures 4 and 6).[69] In general, support preparation is not optimized to maximize the polymeric bed thickness, as just one molecule of the polymer may be enough to immobilize a protein, although this may reduce the impact of the immobilization on the enzyme properties. The fact that this support may rapidly adsorb most proteins in crude extract makes the presence of contaminants detrimental since it may reduce the final loading of the target protein. This occurred when using an excess of total

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protein,[70] and gave an unexpected evolution of the amount of immobilized protein when increasing the amount of offered enzyme.[22] These activated supports have been used in many instances in the preparation of biocatalysts or biosensors. For example, they have even been utilized to immobilize antibodies in biosensors. This application may give freedom of movement to the antibody and avoid steric hindrances for the recognition of the antigen.[71–77] However, considering the huge potential of PEI-coated supports as anion exchangers, it does not look very attractive, as in these cases an inert surface is recommended to prevent false positive.[78–80]

There are many examples in the literature on the use of supports activated only with PEI to immobilize enzymes as biocatalysts.[81-116] Table 1 offers the main reports.

The most important advantage is the simplicity of the immobilization protocol; the process consists in simply mixing the enzyme and the support (usually at pH 7), giving very high immobilization rate, high immobilization yields and expressed activities. However, stabilization of the enzymes is relatively poor compared to the stabilization that can be achieved using covalent immobilization using short spacer arms. An exception is the immobilization of multimeric enzymes (Figure 2 and 3) [24] or stability in the presence of organic solvents (Figure 6). This is recommended mainly for very stable enzymes or when the support is so expensive that it is convenient to reuse it.

The reinforcement of the adsorption may prevent undesired enzyme desorption during operation. To this goal, genetic[117] or chemical[118] enrichment of the enzyme surface in anionic groups has been utilized to strengthen the ion exchange of penicillin G acylase on supports coated with PEI.

This support may be also utilized to co-immobilize several enzymes to catalyze a cascade reaction. That is the case of the coimmobilization of cyclohexanone monooxygenase and glucose-6-phosphate dehydrogenase to transform (2-oxyocyclohexyl)acetic acid into (2-oxooexpan-2-yl)acetic acid.[119]

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In some cases the lay-out of the reactor is very complex and may be a problem if it needs to be dismounted and remounted after enzyme inactivation. In these cases, the reversibility of the immobilization of the enzymes on PEI coated supports become a clear advantage (Figure 9).

One additional problem is that the inactivation of proteins near to the PEI may drive to a fully composites enzyme-polymer where the enzyme maximize the number of enzyme-support interactions(Figure 10) [120,121]. That way, the full desorption of inactivated enzymes immobilized on PEI activated supports may become much harder than the desorption of native proteins. Optimization of this point needs to be considered if the reversibility of the immobilization is a key point to select this protocol.

As stated in the introduction, this ion exchange in a polymeric bed may permit to stabilize multimeric enzymes by involving all enzyme subunits or stabilizing any protein by generating a favorable environment (Figures 2 and 3). However, real rigidification of the enzyme should not be expected (the bonds are weak and the polymer flexible) and the support remains capable of interacting with the protein, which may even produce some enzyme destabilization. [120,121]

3.1.2. Chemical crosslinking of enzyme adsorbed on PEI coated supports

In some cases, to further improve the enzyme properties and fully prevent enzyme desorption, the adsorbed enzymes have been treated with bifunctional chemical reagents, usually glutaraldehyde, to get a covalent immobilization. This immobilization method is very versatile, and some considerations should be brought forward in their use, as the possibility of activating each primary amino group with one or two glutaraldehyde molecules, since this may produce fully different effects.[122–125] This strategy means that reversibility is lost, therefore it must be evaluated whether the gains justify this shortcoming or not. The treatment with glutaraldehyde of the adsorbed enzymes will also crosslink the polymer[124] which will become more rigid and perhaps able to transmit some rigidity

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to the enzyme. Moreover, the enzyme will be modified by glutaraldehyde, in some instances improving the enzyme stability or activity,[44,123,126–128] but in some others it may have highly detrimental effects on enzyme properties. Table 2 shows some examples of the use of glutaraldehyde to prevent enzyme desorption of enzymes immobilized in PEI coated supports[129-143].

Other bifunctional reagents have also been utilized to get this covalent crosslinling, like dimethyl suberimidate (in the immobilization of Jack bean urease).[144] Uridine phosphorylase was immobilized on PEI activated support and further treated with aldehyde dextran to stabilize their multimeric structure, greatly improving its stability.[145] A similar strategy was used later to stabilize the multimeric nucleoside 2′-deoxyribosyltransferase from *Bacillus psychrosaccharolyticus*.[146,147]

In a more sophisticated strategy, polymerization of methyl methacrylate, bromoethyl methacrylate and ethylene glycol dimethacrylate was used to produce a bromide-functionalized matrix.[148] The obtained solid particles were utilized as initiators for the ring-opening polymerization of 2-methyl 2-oxazoline. Finally, the surface brushes were converted into polyethylenimine by acid hydrolysis, activated with glutaraldehyde and used to immobilize α-amylase.[148] The protocol seems to be too complex for industrial implementation, as results did not surpass conventional covalent immobilization. In our view, to use glutaraldehyde or any other crosslinking reagent, will greatly decrease the advantages of PEI, although a higher percentage of the enzyme surface may be involved in the covalent attachment, each bond will have a minimal effect on the enzyme stability due to the polymer flexibility.

3.1.3. Use of PEI supports treated with bifunctional reagents to get a covalent enzyme immobilization

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In some other cases, the support activated with PEI is treated with glutaraldehyde or other reagents before immobilization of the enzyme to directly obtain the covalent immobilization of the enzyme. Considering that this must produce the inter and intramolecular crosslinking of PEI, the advantages of this protocol compared to the use of really rigid flat surfaces activated with similar levels of activation of small amino groups is unclear; perhaps it is not easy to reach similar levels of activation using standard supports compared to the values achieved using PEI. Table 3 shows some of the examples using glutaraldehyde as support activating reagent [149-149-171].

Other bifunctional reagents have been used to activate PEI, like dimethyl sulphate or triethyloxonium tetrafluoroborate to immobilize pectinesterase,[172] epichlorohydrin to immobilize catalase[173] and cholesterol oxidase.[174] Poly-functional aldehyde-dextran has been used to get this covalent crosslinking, like in the case of the nucleoside 2′-deoxyribosyltransferase from *Bacillus psychrosaccharolyticus*.[146] In some cases, unfortunately quite scarce, a comparison among different PEI activation methods is presented. For example, one paper reports that PEI (in some instances partially modified with succinic anhydride) was activated with glutaraldehyde or hexamethylene diisocyanate to immobilize lipase from *T. lanuginosus*.[175] A higher stability was achieved using glutaraldehyde.

In other cases more sophisticated strategies were employed to get covalent attachments. For example, PEI activated supports were treated with epichlorhydrin and then modified with thiophosgene or with succinic anhydride; the carboxyl groups were then converted to hydrazide. These supports were successfully used to immobilize D-glucose oxidase, glucoamylase and cholinesterase (using hydrazide supports the sugar chains of the enzymes were submitted to periodate oxidation).[176] In another case, PEI was modified with aryldiazirine derivatives. After UV irradiation the groups were active (diazirine) and were used to immobilize lysozyme, pepsin, and horseradish peroxidase on

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polypropylene films.[177] The sophistication of these methods makes them complex to implement at industrial level, although they have opened new opportunities should the need arise.

The flexibility of PEI was a key point in the design of different biosensors where the transmission of the electron is a requirement for its proper performance. For example, glucose oxidase and ferrocene were immobilized on PEI to detect glucose.[178] The multifunctionality of PEI is one of the advantages of using this interesting polymer in biocatalysts design, as stepwise modifications are possible.

3.2. Use of PEI to activate nanoparticles to immobilize enzymes

3.2.1. Use of nanoparticles coated with PEI to immobilize enzymes

Immobilization of enzymes in nanoparticles is growing in interest in the scientific literature (Figure 11).[179–181] Non-porous nanoparticles have some advantages, mainly related to the decrease of the diffusional problems as all the enzyme will be on their surface.[23] This makes that this immobilization method may be recommended for using immobilized enzymes versus solid or very large substrates, or even just in the presence of a suspension although the substrate itself may be soluble (e.g., in fruit juice) (Figure 11).[23] In cases where the activity of the enzyme is very high, nonporous nanoparticles prevent the existence of gradients (pH gradients, substrate gradients, even inactivating reagents like H_2O_2 gradients) that can affect the enzyme features,[182–184] not always in a negative way.[22,185] Using porous supports coated with PEI, the cationic polymer could behave like a solid buffer, and could prevent pH gradients formation, but we have not found any report regarding this fact (Figure 12).

However, immobilization in nonporous supports also has disadvantages: the enzyme is exposed to the medium and may be inactivated by proteolysis (catalyzed by protease molecules immobilized on other particles), or it may interact with hydrophobic interfaces (e.g., gas bubbles) (Figure 11), etc.[23]

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Coating with polymers has been proposed to solve this problem, and proteins coated with either aldehyde dextran[186,187] or PEI[188,189] have been proved to be more resistant to this kind of inactivation. Thus, enzymes immobilized inside a polymeric PEI bead could benefit from this protective effect.

The handling of nanomaterials may be complex. This has been solved using para-magnetic materials. Magnetic nanoparticles may be easily recovered from the reaction medium using a magnet and that solves the handling problem of nanoparticles use.[190,191] Moreover, nanomaterials tend to be expensive, and the use of reversible immobilization methods could permit reusing the support. PEI-coated magnetic nanoparticles have been claimed as adequate for enzyme immobilization and present limited environmental impact.[192] Thus, there are many examples of immobilization of proteins in nanoparticles using supports coated with PEI (Figure 11).

The first examples found on this kind of immobilization dated from 2007, as the development of nanotechnology was a requirement prior to this application.[193] Lipases are among the enzymes most usually immobilized on nanoparticles activated with PEI.[194] Superparamagnetic Fe_3O_4 particles coated with PEI were used to immobilize and partially purify lipase from *Candida rugosa*.[195,196] Later, this enzyme was immobilized on zinc oxide (ZnO) nanoparticles with PEI activated with glutaraldehyde and utilized in the synthesis of geranyl acetate.[197] Lipase from *T. lanuginosus* was immobilized on polyethyleneimine-modified superparamagnetic Fe_3O_4 nanoparticles and further crosslinked using glutaraldehyde.[198] This enzyme was later immobilized in a similar support assisted with divalent metal chelated ions.[199] Lipases have been immobilized on PEI-modified magnetic nanoparticles and used to produce vitamin A palmitate via esterification reactions.[200] Fe_3O_4 magnetic nanoparticles were coated with PEI followed by grafting β-cyclodextrin and utilized to immobilize lipase utilized in the production of ethyl valerate.[201] Other enzymes have also been immobilized on PEI coated nanoparticles. β-galactosidase from *Kluyveromyces fragilis* has been immobilized on magnetic poly(glycidyl methacrylate-ethylene glycol dimethacrylate-hydroxyethyl methacrylate) nanospheres

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grafted with polyethyleneimine.[202] α-rhamnosyl-β-glucosidase from *Acremonium sp.* DSM 24697 was immobilized by ion exchange and cross-linking onto PEI-iron particles, improving the results reported using polyanilide.[203] Partially phosphonated polyethylenimine has been used to coat iron oxide nanoparticles and used to covalently immobilize trypsin and used in proteomic analysis.[204] In another paper, metal ion-chelated linear PEIs coating nanoparticles were used to immobilize and stabilize glycerol dehydrogenase.[205] Laccase has also been immobilized on magnetic Fe_3O_4 nanoparticles modified with polyethylenimine.[206] Cellulose nanocrystals were successfully combined with PEI modified Fe_3O_4 nanoparticles and successfully used for the immobilization of papain.[207] Acetylcholinesterase has been immobilized on PEI-coated silica nanoparticles improving its stability.[208]

3.2.2. Use of PEI to form the nanoparticles to be used as carries of enzymes

In some instances, PEI is not only used to immobilize the enzyme, but also plays a fundamental role in nanoparticle building. PEI is in many cases used as template and reducing agent in the production of nanoparticles. Thus, PEI has been used as template for the formation of silica nanoparticles trapping chemically modified anionic peroxidase, showing direct electron transfer at 0 mV versus Ag/AgCl.[209] Amine dehydrogenase was immobilized on hybrid titania nanoparticles, which were produced by polyethylenimine coating and templated biomineralization.[210]

Other examples where the role of PEI in the nanoparticle may go beyond immobilization of enzymes is the formation of colloidal stable nanoparticles using mixtures of PEI and anionic polymers. For example, mixtures of PEI and poly(maleic acid-co-propylene) have been used to form stable colloidal nanoparticles, crosslinked via maleic anhydride and utilized to immobilized laccase.[211]

Some sophisticated strategies have been used, based on PEI properties to bind the nanoparticles to other solids. For example, submicron-sized poly(N-isopropyl acrylamide)/polyethyleneimine core-

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shell microgels were prepared in aqueous media using tert-butyl hydroperoxide as an initiator followed by the formation of nanoparticles on the surface of the microgels.[193] PEI was used as binder for the assembly of the gold nanoparticles/microgel complex, and finally horseradish peroxidase and urease were successfully immobilized.[193] In another paper, bacteria cellulose nanofiber nanocomposites were produced and coated with gold nanoparticles using PEI as reducing and linking agent.[212] Then, this composite was used to immobilize horseradish peroxidase. A similar protocol was utilized later to prepare a support where glucose oxidase was immobilized using carbodiimide as enzyme linking agent in this case.[213] Gold nanoparticles were also immobilized on the inner wall at the inlet end of a capillary electrophoresis unit treated with PEI; and then used to immobilize L-glutamic dehydrogenase.[214] This immobilized enzyme was used as a sensor for enzyme inhibitors. The immobilization of multi-walled carbon nanotubes using nafion and polyethylenimine as dispersants and glucose oxidase as enzyme was utilized to improve the determination of glucose.[215] Self-polymerization of dopamine on the surface of $CaCO_3$ particles was used to form polydopamine and used as an adhesive layer to bind PEI through Michael addition reaction or Schiff base reaction. Then, titania was generated and deposited on the surface of the PEI nanoparticles via bioinspired mineralization process initiated by the free amine groups of PEI. EDTA treatment produced hybrid microcapsules by eliminating the $CaCO_3$ templates and used to immobilize catalase.[216] Gold nanoparticles were *in situ* grown on graphene oxide surface using polyethylenimine as reducing and stabilizing reagents, and then it was used as support for aptamer immobilization to detect human α-thrombin from human serum.[217]

3.2.3. Use of nanoparticles coated with PEI as biosensors.

In many instances nanoparticles have been utilized in biosensor design, where the required amount of support is far lower than that in biocatalysis design. For example, glucose oxidase has been immobilized on nanotubes, using PEI to adsorb the enzyme and covalently immobilize ferrocene

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derivatives to develop a glucose sensor (Figure 13).[218] Laccase has been immobilized on gold nanoparticles coated with PEI and used as biosensor.[219] Lactate oxidase was covalently immobilized on platinum nanoparticles supported on graphitized carbon nanofibers using PEI and glutaraldehyde (GA).[220]

3.3. Use of PEI to coat cell wall to co-immobilize enzymes and cells

Coimmobilization of enzymes and cells has significant interest. For example, glucose oxidase was immobilized on PEI-coated yeast cells and attached to cotton thread (Figure 14). The crosslinking with glutaraldehyde avoided undesired enzyme release and permitted several reuses without losing enzyme activity.[221]

In another paper, PEI was used to form PEI- D-lactate dehydrogenase - *Candida boidinii* aggregates that were later adsorbed onto cotton cloth.[222] That way, D-lactate dehydrogenase (LDH) was used to transform 2-oxo-4-phenylbutyric acid to R-2-hydroxy-4-phenylbutyric acid oxidizing NADH to NAD+, which was regenerated by the formate dehydrogenase presented in the cells of *Candida boidinii*.

34. Use of PEI to activate complex reactors to immobilize enzymes in the reactor itself

The immobilization of enzymes on complex reactors makes mounting and dismounting of the reaction complex (Figure 9). Thus, the use of some reversible immobilization enzyme protocol to prevent the necessity of reactor modification becomes a requirement. This might be exemplified in the use of monoliths (Figure 9), a new support configuration that is receiving increasing attention in biocatalysis [223–229] and chromatography.[230,231] Monoliths have better mass transfer hydrodynamics than

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their standard particulate counterparts,[232] becoming very useful systems to treat high volumes of substrate. However they require a correct layout to ensure the correct circulation of the substrate, avoiding the promotion of preferential ways, considering that they are adequate when high substrate flow is utilized.

The activation of complex reactors with PEI is a good alternative for enzyme immobilization. Lactase from *Aspergillus oryzae* was immobilized on high-porosity mullite advanced ceramic material coated with PEI,[233] showing that the open microstructure of the monoliths permitted a good accessibility to the enzyme molecules immobilized inside the walls of the monolith and permitted high enzyme loadings. Later, classical cordierite and acicular mullite (ACM) monoliths were coated with silica and functionalized with PEI using different protocols. β-galactosidase from *Aspergillus oryzae* was then immobilized on them.[234] An extracellular lipase from *Yarrowia lipolytica* was one-step immobilized and purified from lipases from *Candida* sp. using poly(glycidyl methacrylate-co-ethylene glycol dimethacrylate)monolithic columns functionalized with PEI.[235] Four lipase isoforms from *Candida sp.* 99-125 were separated (isoform A, isoform B, isoform C and isoform D).

Acetylcholinesterase and choline oxidase were co-immobilized on a monolith and used for the evaluation of acetylcholinesterase inhibition caused by diverse agents.[236] Cryogels with high catalyst density were produced through cryostructuration of whole *Escherichia coli* cells (forming a monolith) containing β-glucosidase and crosslinking PEI and polyvinyl alcohol activated with glutaraldehyde.[237]

Biomimetic silicification was used to trigger enzyme immobilization on the surface of the graphene oxide nanosheets/FeOOH hydrogel for constructing monolithic biocatalytic reactors.[238] The graphene oxide adsorbs PEI and this polymer is used as the mineralization-inducing agent, forming silica on the graphene oxide surface. Here, penicillin G acylase was trapped simultaneously. This monolithic system displayed simple recyclability and high stabilities.

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On-chip glucose oxidase and choline oxidase immobilized monolith micro-reactors were used to analyze the kinetic features of the enzymes.[239] The monolith was produced employing a sol-gel method, which was later functionalized using PEI. Polycarbonate microchannels supports were activated with PEI and used to immobilize alkaline phosphatase.[240] A glucose oxidase reactor constituted by a microchannel assembled in poly(methyl methacrylate), coated with PEI, and connected to an amperometric detector which was used to quantify glucose.[241] In another paper, the same group used different materials to immobilize glucose oxidase in microchannels using PEI activated with glutaraldehyde.[242] Later, microreactors with immobilized ascorbate oxidase, catalase, glutamate dehydrogenase and a coimmobilized glucose oxidase and horseradish peroxidase were immobilized using similar strategies.[243]

Similarly, PEI has been used to activate membranes to immobilize enzymes in membrane reactors. For example, lipase B from *C. antarctica* has been immobilized in an α-alumina ceramic support activate with gelatin-PEI and successfully utilized in the production of butyl butyrate.[244]

PEI has also been employed to immobilize proteins in chips for ELISA assays even though these supports, as commented before, will have a strong capacity as anion exchangers. Trace levels of α-fetoprotein (hepatocellular carcinoma biomarker) have been detected using a monoclonal antibody covalently immobilized on the amino groups of the PEI using poly(methyl methacrylate) microfluidic chips and electrochemical detection systems.[245] Enzyme microencapsulation (Figure 15) has been frequently used to immobilize enzymes, in many instances used later in a microreactor. For example, laccase was encapsulated via interfacial cross-linking of PEI and packed into a capillary-sized reactor with off-line capillary electrophoresis for substrate quantification.[246]

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A mixture of lactate oxidase with PEI and poly(carbamoyl)sulphonate hydrogel was used for enzyme immobilization onto a platinum disk of a transducer in a system developed for development of L-lactate determinations.[247]

4 Use of PEI as glue to immobilize multilayers of enzymes

4.1. Use of PEI to form multilayers of the same enzyme

The formation of enzyme multilayers may permit increasing enzyme loading on a support (Figure 7). This may be interesting using porous supports, but becomes critical when the enzyme is immobilized on macroscopic non-porous supports having low specific area. The main point to be considered in order to succeed is to keep the enzyme activity of the in-between layers. To do this, enzyme distortion and diffusional problems need to be kept to the minimum. PEI, a random coil and flexible polymer, may be a very good alternative to act as glue between enzyme layers, or any other molecule. As previously discussed, the flexible structure permits to induce scarce alterations on the protein, and if a protein is coated with PEI and then another protein molecule is immobilized, this second protein will not interact with the first protein, but with PEI.

Glucose oxidase is one of the most widely used enzymes for this strategy. For example, PEI and poly(dimethyldiallyl- ammonium chloride) were assembled in alternate layers to achieve molecular films, without influence from substrate diffusion at up to 5 μg of immobilized enzyme in 50 m^2.[248] The enzyme film was also prepared by a premixing method, in which a glucose oxidase polyion complex was assembled alternately with another oppositely-charged polymer. This permitted to increase by 67-folds the activity compared to the conventionally assembled films. In another paper, multilayers of glucose oxidase using different ionic polymers as glue were produced onto a thiolated-gold surface and the resulting bioelectrode was used for glucose biosensing.[249] The supramolecular

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structure was prepared by gathering polyethylenimine and Nafion (as anti-interference barrier), followed by adsorption of DNA (as stabilizing layer) and polyethylenimine and finally by the alternate deposition of polyethylenimine and glucose oxidase (as the biocatalytic film).[249] Glucose oxidase was also immobilized on multiwall carbon nanotube-modified glassy carbon electrode, using a layer of PEI to immobilize the enzyme. For multilayers, the next layer of PEI was directly assembled on the enzyme.[250]

Galactooligosaccharides were produced from lactose using β-galactosidase from *Aspergillus oryzae* immobilized on cotton cloth via PEI as biocatalyst - enzyme aggregate formation and growth of aggregates on individual fibrils of cotton cloth leading to multilayer immobilization of the enzyme.[251] In this case, 250 mg of enzyme per g of support could be immobilized. Later, a similar protocol was used using as support low-pressure plasma-modified cellulose acetate membrane.[252] Lactase was also covalently attached to low-density polyethylene for in-package production of lactose-free dairy products.[253] Multilayers of the enzyme were immobilized alternating polyethylenimine, glutaraldehyde and lactase (the 5-layer sample reaching up to 1.3 μg lactase/cm^2), although the affinity of the immobilized lactase towards the substrate remains unchanged after immobilization, the k_{cat} showed a decrease with each layer of attached lactase. In our opinion, this may be due to glutaraldehyde modification of the enzyme.

Urease has been immobilized on the walls of silicon microchannels by alternating layers of polycationic polymers (PEI, polydiallyldimethylammonium or polyallylamine combined) and polyanions (polystyrenesulfonate or polyvinylsulfate).[254] The best results were obtained using PEI/polystyrenesulfonate/PEI/urease/PEI (repeating this model for more layers). This strategy was used later by the same group to immobilize organophosphorous hydrolase on the same support.[255]

Single polycation cross-linked multilayers, based on polyethyleneimine, have been prepared using a method of 3,3′,4,4′- benzophenonetetracarboxylic-dianhydride (BTCDA)-mediated

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electrostatics and hydrogen bonds layer-by-layer assembly.[256] Different PEI polymers were adsorbed onto silica microparticles or silicon wafers, crosslinked using 3′,4,4′- benzophenonetetracarboxylic-dianhydride (that introduced carboxylic acids to the polymer), and used to adsorb a new layer of PEI. This was later used to immobilize pepsin or lysozyme using glutaraldehyde.

Lipase from *T. lanuginosus* was immobilized into multiple layers using polyethylenimine-treated cotton flannel cloth,[257] utilizing the enzyme property of forming bimolecular aggregates.[258,259] The use of aggregated enzyme permitted to increase the activity by 30 %.[257]

Nanostructures may also be glued with PEI forming multilayers. For example, microcylinders of halloysite of 50 nm diameter, 500 nm length and with 20 nm diameter hollow inner core were assembled by alternate adsorption with PEI, yielding ordered multilayers containing up to 20 layers of tubules connected by PEI.[260] The immobilization of alcohol dehydrogenase and halloysite loaded with NADH in alternation with PEI was achieved, providing a direct supply of NADH to the enzyme.

Thus, PEI has revealed itself as a powerful tool to produce many layers of enzymes keeping good activity even on the enzymes immobilized near the support surface.

4.2. Use of PEI as glue to co-immobilize enzymes.

This strategy has been also used to co-immobilize two enzymes that may be coated with PEI and poly(styrenesulfonate). For example, layers of glucoamylase and glucose oxidase were assembled on an ultrafilter by layer-by-layer adsorption using PEI as glue.[261] That way, starch was converted to gluconic acid and H_2O_2. The optimal global activity was obtained when the glucoamylase layer was deposited on the layer of glucose oxidase.[261] In another paper, using alternating layers of PEI and poly(styrenesulfonate), a multilayer of alkaline phosphatase and glucose oxidase were formed,[262] although enzyme coimmobilization does not appear really advantageous in this case.[23]

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In another research, a layer of poly(styrene sulfonate) was formed by adsorption on the surface of several supports (platinum electrode, glass plate, quartz crystal microbalance, quartz plates, mica and silicon substrates) followed by a layer of PEI.[263] This multilayer strategy was used to trap cholesterol esterase and cholesterol oxidase to detect cholesterol. In another paper, the enzymes lactate oxidase (to detect lactate) and ascorbate oxidase (to eliminate interferences with ascorbic acids) were co-immobilized on Pt electrodes with Ceria nanoparticles (as an oxygen deposit) using PEI as immobilizing agent and using several layers of each enzyme.[264] The final configuration of the biosensor was: Pt/CeO 2–3 layers of PEI/Lactate oxidase-3 layers of PEI/ascorbate oxidase.

In the examples listed above all enzymes were co-immobilized via ion exchange. That means that when the least stable enzyme is inactivated, both enzymes need to be discarded,[23] being this one of the frequently overlooked problems of enzymes coimmobilization. However, a strategy has been very recently proposed that involves the formation of layers of the involved enzymes using PEI as glue between one enzyme and the other, to prevent this coimmobilization drawback (Figure 16). The strategy was proposed to solve this problem in one specific situation, when one of the enzymes can be immobilized via ion exchange, and, at the same time, cannot be highly stabilized via multipoint covalent attachment. Moreover, this immobilized enzyme should be less stable than the other immobilized enzyme. The proposal was to immobilize a lipase via interfacial activation on octyl-agarose beads,[31] to coat the enzyme with PEI, and then, to immobilize the second enzyme on this PEI layer (Figure 16).[265] After inactivation of the enzyme, it could be desorbed and the lipase could remain immobilized and active on the support. It was found that enzyme desorption was not so simple, as the inactivated enzyme maximized the enzyme-polymer interactions.[120,121] As some PEI was desorbed when the second enzyme was released, the immobilized enzyme required to be incubated with PEI before immobilizing a new batch of the second enzyme. This problem is not relevant at laboratory

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scale but it is quite relevant at industrial level. It has been partially solved using octyl-glyoxyl agarose and modification of the lipase with glutaraldehyde.[266]

5. Use of PEI to enhance the formation of solid supports formed by ionic polymers

There are many examples in the literature where PEI is not used to activate supports, but to improve the physical stability of supports formed by interaction between anionic polymers and divalent cations, mainly to trap enzymes. Trapping of enzymes should not improve enzyme rigidity, but may generate an adequate enzyme environment or prevent subunit dissociation of multimeric enzymes.

One example is the case of supports formed by alginate or κ-carrageenan and calcium.[267] Although most examples use chitosan to reach this goal,[268] some examples may be found using PEI to stabilize this kind of supports and used to immobilize D-hydantoinase[269] or penicillin acylase.[270] Moreover this modification allows introducing glutaraldehyde groups on the support (via the primary amino groups of PEI), and prevents enzyme release, a usual problem using trapping techniques for enzyme immobilization.[271] Some examples may be found in the literature of this use of PEI, for example the immobilization of lipase from *Expansum penicillium* to be used in the esterification of 1-dodecanol with dodecanoic acid in benzene,[272] porcine pancreatic lipase to hydrolyze olive oil,[273] β-glucosidase from *Aspergillus niger* to produce reducing sugar from algae cellulosic residue.[274] Calcium pectinate beads were treated with PEI and glutaraldehyde and used to immobilize β-D-galactosidase.[275]

In other instances, PEI is utilized as nucleation point to get a solid support. Several enzymes have been trapped by silica co-precipitation. The process consists in a mixture of enzyme and PEI, and the further incorporation of a phosphate-buffered silicate solution. This formed a colloidal co-

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precipitate consisting of the enzyme and PEI within a hydrated, amorphous silica matrix.[276] Later a similar strategy was used to coat the surface of the immobilization support with silicic acid, and later used to immobilize several proteins via ion exchange.[277]

In another paper, N-isopropylacrylamide, N,N-methylene bis-acrylamide and PEI were utilized to form a microgel via adsorption.[278] This microgel was used to immobilize acetyl CoA synthetase, and finally the microgel was covalently immobilized upon polyethylene terephthalate track etched membrane.[278]

Other research shows a method based on polyphenol chemistry for the facile preparation of microcapsules in four steps: production of a sacrificial template, assembly of the polyphenol coating on the template surface, cross-linking of the polyphenol coating by cationic polymers (PEI), and removal of the template.[279] The strong interfacial affinity of tannic acid helped towards the generation of polyphenol coating through oxidative oligomerization, while the reactivity of tannic acid permitted the cross-linking with PEI through Schiff base/Michael addition reactions. Glucose oxidase was immobilized in the microcapsules, showing improved pH and thermal stabilities and good catalytic activity.[279]

6. Use of PEI capsules to immobilize enzymes

Encapsulation of enzymes inside PEI capsules has been described as a very efficient way to avoid dissociation of multimeric enzymes (Figure 3). For example, glutamate dehydrogenase from *Thermus thermophilus* and formate dehydrogenase from *Pseudomonas* sp. were coated with PEI and treated with glutaraldehyde to prevent enzyme dissociation under acidic pH value.[188] This permitted to have a free enzyme where dissociation was not possible, enlarging the range of conditions where the enzyme may be handled, in operation or just to later immobilize the composite.

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The enzyme-PEI composite was ionically exchanged on cationic exchangers. For example, formate dehydrogenase could be treated with PEI and glutaraldehyde after adsorption on cationic exchangers, where the native enzyme cannot become immobilized. Later, a similar strategy was used to stabilize glutamate dehydrogenase from *Escherichia coli*, showing that together with preventing enzyme dissociation, this strategy also improved enzyme stability in stirred systems.[189]

Glucose oxidase was coated with PEI and this stabilized enzyme/polymer composite was immobilized into woodceramics that served as both immobilization matrix and electrochemical transducer.[280] Again, immobilization was only possible after coating the enzyme with PEI to generate a cationic enzyme surface. A cross-linked PEI wall was generated from an emulsion of an aqueous phase containing laccase, forming microcapsules that were later immobilized on the surface of the biosensor electrode.[281,282] The laminar jet break-up technique was used to generate PEI microcapsules for immobilization of glucose oxidase from *Aspergillus niger* and laccase from *Trametes versicolor*.[283] Later, these enzyme/PEI microcapsules were used to immobilize the enzymes in paper.[284] In another research effort, ink was supplemented with microcapsules produced via interfacial polycondensation of PEI with the sebacoyl chloride and containing laccase from *Trametes hirsuta* or *Trametes versicolor*.[285] Then, they were printed or coated on a paper substrate.

In another paper, an aqueous solution of PEI was mixed with an aqueous solution of Fmoc-diphenylalanine peptide and sodium silicate.[286] This way a new class of organic-inorganic hybrid capsules (FPSi) with multi-layered structure was fabricated and used to encapsulate epoxide hydrolase and bovine serum albumin.

7. Use of PEI in the preparation of crosslinked enzyme aggregates (CLEAs)

The immobilization of enzymes by CLEAs technology was proposed by Prof Sheldon´s group.[287,288] This protocol was a simplification of the enzyme crosslinked crystals,[289,290] consisting in

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the precipitation of the enzymes (adding some precipitant agent) and their further crosslinking (usually with glutaraldehyde, although in some instances some alternatives have been used).[291,292] That way the enzyme aggregate cannot solubilize when the precipitant agent is removed (Figure 17). Advantages involve that enzyme purity is not a requirement, and aggregation is far simpler than crystallization. Thus, combi-CLEAs have been produced by co-aggregating several enzymes.[293–295] However, in some instances the crosslinking step may become a problem if the target protein is poor in Lys groups in the surface. This may be solved using bovine serum albumin or other protein feeder,[296–298] or if the enzyme could be chemically aminated.[299] As all the solid is enzyme, diffusion problems tend to be high:[23] this may be partially reduced using an inert protein.[300]

PEI may play a significant role in facilitating the crosslinking of aggregates, as it is very rich in primary amino groups, and also reduces the protein density decreasing substrate diffusional limitations. Thus, stable CLEAS of glutaryl acylase,[301] lipases from *Alcaligenes sp*., *C. antarctica* (fraction B), *Geotrichum sp.* and *Serratia marcescens*[302–304] could be prepared using PEI as a feeder while the direct crosslinking failed. In the case of lipases, enzyme features (e.g., specificity) could be also tuned using PEI.[302]

The cationic nature of PEI has been used to generate "saline" environments (Figure 6), and has improved the stability of CLEAs of oxygen-labile nitrilases versus oxygen[305] or penicillin G acylase versus organic solvents (mixing PEI and dextran sulfate).[306] These penicillin acylase preparations were used in the synthesis of diverse antibiotics.[307–309]

8. Modification of immobilized enzymes with PEI

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The points above showed examples where PEI was used to activate the support or to form the own support. Now, we will give a view on the employment of PEI to modify previously immobilized molecules to improve different features, from stability to specificity or activity.

8.1. Intersubunit crosslinking of multimeric enzymes by PEI immobilization or PEI modification of immobilized enzymes

The first step in the inactivation of multimeric enzymes involves in many instances dissociation of the enzyme.[310–312] The simultaneous immobilization of all enzyme subunits on a support prevents this inactivation cause (Figures 2 and 3). However in some instances the geometry of the enzyme prevents involving all enzymes in the immobilization.[24] For these instances, the coating of the enzyme with a polymer has shown to be a powerful tool to crosslink all enzyme subunits, although in many instances aldehyde dextran has been utilized.[313,314] In fact, in some instances enzymes immobilized on PEI coated supports have been further stabilized by treatment with aldehyde dextran. Nucleoside 2′-deoxyribosyltransferase *Bacillus psychrosaccharolyticus* was immobilized on PEI coated agarose beads followed by crosslinking with 70% oxidized dextran.[147] This biocatalyst was later used to produce trifluridine.[146] A similar strategy was utilized with a pyrimidine nucleoside phosphorylase from *Bacillus subtilis* and a thymidine phosphorylase from *Escherichia coli*.[315] The biocatalysts were used in the synthesis of 5-halogenated pyrimidine 2′-deoxyribonucleosides by transglycosylation. Nucleoside phosphorylases from *Citrobacter koseri*, *Clostridium perfringens* and *Streptococcus pyogenes* were also immobilized on PEI coated supports and crosslinked with partially oxidized aldehyde dextran.[316]

The homodimeric thymidine phosphorylase from *Escherichia coli* was immobilized via ion exchange on PEI coated agarose and Sepabeads ® and cross-linked with partially oxidized dextran

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aldehyde to prevent enzyme dissociation.[317] These biocatalysts were employed for the one-pot synthesis of 5-fluoro-2′-deoxyuridine starting from 2′-deoxyuridine and 5-fluorouracil. Uridine phosphorylase from *Bacillus subtilis* was immobilized/stabilized onto Sepabeads coated with polyethyleneimine and cross-linked with aldehyde dextran (UP-Sep-PEI-Dx).[145] This biocatalyst was used in combination with immobilized purine nucleoside phosphorylases in the synthesis of 2′ -deoxyguanosine by enzymatic transglycosylation in aqueous solution between 2′-deoxyuridine and guanine.[318]

In another example, lipase dimers were immobilized on PEI-coated supports. Lipases have a certain tendency to form bimolecular aggregates confronting the open form of two lipase molecules with clearly altered functional properties, and their immobilization in PEI coated supports seem to keep these dimers.[258]

However, there are a handful of examples of the use of PEI as the final crosslinking agent. For example, the tetrameric β-xylosidase from *Selenomonas ruminantium* was immobilized on glyoxyl agarose beads, with a slight stabilization due to the non-implication of all enzyme subunits.[319] The modification of the immobilized enzyme with aldehyde-dextran or polyethylenimine fully stabilized the quaternary structure of the enzyme and greatly improved enzyme stability. Another paper shows that the immobilization of the multimeric alcohol dehydrogenase from baker's yeast on agarose activated with glyoxyl groups stabilized the enzyme by 50-folds.[320] However, not all enzyme subunits become immobilized. Physical modification of the immobilized enzyme with PEI permitted to crosslink all enzyme subunits, improving enzyme stability and preventing enzyme dissociation.

In another paper, the heterodimeric cephalosporin C acylase was ionically exchanged in the aminated support LX1000-HA, treated with glutaraldehyde and later treated with PEI to improve the enzyme stability.[321]

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Thus, the large size of PEI may be a very useful way to physically crosslink multimeric enzymes. However, it may be expected that this protection is only shown under conditions where PEI remains interacting with the enzyme (that is, it may be lost at high ionic strength or extreme pH values). Treatment of the modified enzymes with glutaraldehyde or other bifunctional reagents may make this strategy useful under all conditions, but it is not usually performed.

8.2. Generation of hydrophilic environments by PEI coating

The generation of hydrophilic environments (Figure 4) may be used to partition some detrimental hydrophobic compounds from the enzyme environment (Figure 6) and thus, to improve enzyme performance.

A general procedure for stabilization of O_2-labile enzymes exploiting "salting out" of oxygen from highly hydrophilic enzyme microenvironment was proposed by Klibanov`s group a long time ago, suggesting that PEI could be a very suitable support activating agent for this goal.[69]

The generation of highly hydrophilic environments surrounding enzyme molecules has been proposed as a strategy to produce a partition of hydrophobic organic solvents and that way reduce their concentration in the enzyme surrounding (Figures 4 and 6). This has been applied to achieve penicillin G acylase immobilized biocatalyst that coupled a high rigidification by immobilization via multipoint covalent attachment to a highly hydrophilic environment formed by “solid salts” formed by aldehyde dextran and PEI-dextran sulfate layers, and that were highly stabilized in the presence of organic solvents.[322,323] These biocatalysts could be used in the hydrolysis of penicillin G in the presence of organic solvents[324] in the thermodynamically controlled synthesis of antibiotics,[309,325] or other amides,[326] or in the enantioselective synthesis of phenylacetamides.[327]

The coimmobilization of penicillin G acylase and PEI did not improve the stability of the immobilized enzyme. This was attributed to the fact that the enzyme was not adsorbed on PEI coated

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supports,[328] therefore the polymer did not really prevent the exposition of enzyme to high organic solvent concentrations due to the lack of enzyme-polymer interactions. The genetic introduction of eight additional Glu residues homogenously distributed throughout the enzyme surface permitted the polymer adsorption, and thus enzyme stabilization versus organic solvents by coimmobilization of the enzyme and the polymer.[329]

In another paper, lipases from *Rhizomucor miehei* and *Candida rugosa* were immobilized on Sepabeads decaoctyl and coated with PEI to decrease the interactions between the immobilized lipase molecules and the organic solvents when using these derivatives in anhydrous media.[330] These biocatalysts were more active and stable than the uncoated preparations when catalyzing esterifications and transesterifications in anhydrous media. In another instance, the coating of a commercial preparation of lipase B from *C. antarctica* (form B) (Novozym 435) with PEI improved their stability in the production of structured lipids, mainly by preventing the formation of crystals of aggregated material and improving the support stability.[331,332] This use of PEI has been scarcely explored although it is a simple way to protect enzymes from hydrophobic compounds.

8.3. Physical crosslinking of reversibly immobilized enzymes

Physical immobilization of enzymes is very popular, because it is very simple to implement, the supports tend to be stable, and the immobilization is reversible, enabling the reuse of the support when the enzyme has been inactivated. This immobilization is usually not very efficient in improving enzyme stability, except when multimeric enzymes are involved. However, there is a case where the physical immobilization produces a high stabilization of the enzyme: the immobilization of lipases on hydrophobic supports.[333] This stabilization is even higher than using multipoint covalent immobilization,[32,33] because the lipase interfacially activated versus the hydrophobic surface of the

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supports gives a lipase form that is more stable than the enzyme in closed/open conformation, even if this form is stabilized via multipoint covalent attachment [34,334,335]

However, lipases are desorbed from the hydrophobic support under drastic conditions,[336] or even by the action of some substrates with detergent properties[35,36,337] producing product contamination by the enzyme and biocatalyst inactivation. This has been solved using heterofunctional supports combining acyl moieties to achieve interfacial activation of the lipase and covalent reactive groups.[336,338–342] Also, covalent crosslinking has been performed using aldehyde dextran.[343] A simpler solution has been recently proposed: the coating of the fully loaded enzyme preparations with PEI and dextran sulfate greatly reduced enzyme desorption and significantly improved enzyme stability (Figure 18). Thus, some lipases immobilized on octyl agarose, like those from *Rhizomucor miehei*,[344] *C. antarctica* (form B),[345,346] *T. lanuginosus* or the phospholipase Lecitase have been stabilized by coating with PEI.[347] That wat, coating of octyl-lipase preparations with PEI permits to reduce enzyme leakage without losing the reversibility of the immobilization of the lipases, and the positive effects of stabilization versus hydrophobic molecules may be also achieved.

8.4. Bioimprinting of enzyme features

Enzyme bioimprinting may be defined as a strategy to maintain an enzyme conformation induced by a given agent in the absence of this particular agent.[348–350]

PEI or supports coated with PEI have proven to be very efficient for this goal. For example, the activity of Lecitase Ultra covalently immobilized on cyanogen bromide was increased if incubated in the presence of some detergents. [351] However, the detergents were negative for enzyme stability.[351] To keep this hyperactivation, which was associated to the stabilization of the open form of the enzyme, the immobilized enzyme was incubated with PEI in presence of sodium dodecyl sulfate, achieving a

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Lecitase form 50-folds more active than the initial preparation. It was showed that the PEI treatment maintained the open form of the lipase in absence of the detergent (Figure 19).[351]

Immobilization of lipase from *C. antarctica* (form B) on PEI supports at different pH values permitted to keep the properties exhibited by the enzyme under the immobilization pH values in the resolution of R,S-mandelic acid methyl ester, even though the reaction was performed at other pH values.[352]

Finally, the oligomeric state of invertase from *S. cerevisiae* depends on the pH, presenting different properties.[353,354] The enzyme was immobilized on Sepabeads coated with PEI at different pH values where the aggregation of the enzyme was different. These biocatalysts presented different stabilities, suggesting that the immobilized enzyme kept this aggregation state under any pH value.[355]

8.5. Modification of enzyme properties

It has been reported how different immobilization strategies or the further chemical or physical modification may be used to tune enzyme selectivity, specificity or activity.[22,23,45,46] In many examples, PEI-coated supports or modification with PEI of immobilized preparations (Figure 5) have been used to achieve this alteration in enzyme features.

In some cases, PEI was used to modify the properties of immobilized enzymes. Lecitase immobilized in cyanogen bromide agarose or on octyl-agarose and further coated with PEI permitted to significantly increase enzyme activity (even by 30 folds using the octyl-Lecitase-PEI at pH 5 in the hydrolysis of methyl phenyl acetate).[356] Lipases from *C. antarctica* (form B), *T. lanuginose* and *Rhizomucor miehei* were immobilized on CNBr-activated Sepharose beads or onto octyl-agarose and used in the hydrolysis of sardine oil and submitted to different chemical or physical modifications,

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among them with PEI.[357] The selectivity and activity of lipase from *T. lanuginose* immobilized on octyl-agarose increased two-fold after PEI modification.

In other cases, immobilization of the lipases on PEI-coated supports enables the alteration of their catalytic properties. Lipase B from *C. antarctica* was immobilized on different supports and used in the hydrolytic resolution of several (±)-α-hydroxy-phenylacetic acid derivatives.[358] The enzyme immobilized on cyanogen bromide presented an enantiospecificity of 7.4, while the enzyme immobilized in PEI coated agarose exhibited an enantiospecificity of 67 in the hydrolysis of α-hydroxy-phenylacetic acid methyl ester under similar conditions. Moreover, the enantioselectivity of the PEI derivative decreased from 67 to 14 when the temperature went from 25 to 4°C at pH 5, while the E of some other derivatives improved significantly under similar experimental changes.[358]

The enantiospecificity of the lipase from *Candida rugosa* in hydrolysis of R,S methyl mandelate went from <2 using some preparation to around 200 for the towards S-isomer using a lipase immobilized on PEI coated support.[359]

The lipase from *Aspergillus niger* immobilized on PEI coated support was six times more active than a covalent preparation in the hydrolysis of 1-thioisopropyl-2,3,4,6-tetra-O-acetyl-β-d-galactopyranoside. However, this covalent preparation was more active in the hydrolysis of galactal.[360] The lipase from *Candida rugosa* immobilized on octyl agarose was completely specific and regioselective in the hydrolysis of galactal, producing the C-6 monohydroxylated product in 99% yield while the enzyme immobilized on PEI coated agarose beads hydrolyzed in C-6 but also in C-3 positions.[360] Lipase from *Aspergillus niger* immobilized on supports coated with PEI was 800 times more active than the enzyme immobilized on octyl-agarose in the hydrolysis of 2-acetamido-2-deoxy-1-(4-nitrophenyl)-3,4,6-tri-O-acetyl-β-d-glucopyranoside.[361] However, the octyl derivative was five times more active than the PEI preparation in the hydrolysis of 1-(4-nitrophenyl)-2,3,4-tri-O-acetyl-β-d-xylopyranoside.

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Lecitase immobilized on PEI coated supports was much more active in the hydrolysis of 1,2,3,4,6-penta-*O*-acetyl-α-d-mannopyranose or 2-acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose that the enzyme immobilized on octyl-agarose, but it was 4 folds less active in the hydrolysis of 1,2,3,4,6-penta-O-acetyl-β-d-galactopyranose.[362]

Among the different enzymes and immobilized biocatalysts, lipase from *T. lanuginosus* immobilized on supports coated with PEI was the most efficient catalyzing the hydrolysis of 1,2,3,4,6-penta-O-acetyl-β-D-glucopyranose, permitting us to obtain up to 70% of the 6-hydroxy product.[363] In the hydrolysis of 2-acetamido2-deoxy-l,3,4,6-tetra-0-acetyl-β-D-glucopyranose, the form B of the lipase from *C. antarctica* immobilized on octyl agarose was not regioselective. However, the enzyme immobilized on supports coated with PEI was highly specific and regioselective producing the 6-hydroxy-2- acetamido-2-deoxy-l,3,4-tri-O-acetyl-β-D-glucopyranose in 70 % yield.[363] A lipase from porcine pancreas was adsorbed on PEI-coated support and utilized in the resolution of (±)-glycidyl butyrate, showing a enantiospecificity of 6.[127] The treatment of the adsorbed lipase with glutaraldehyde permitted to increase this value to E = 61. The use of lipase B from *C. antarctica* immobilized on a support coated with PEI permitted to obtain (S)-glycidyl butyrate (90% ee), while other immobilized preparations offered much lower values.[364]

A contaminant esterase contained in commercial extracts of *C. antarctica* (form A) was immobilized on supports coated with PEI and used in the enantioselective hydrolysis of (3RS,4RS)-trans-4-(4′-fluorophenyl)-6-oxo-piperidin-3-ethyl carboxylate hydrolyzing mainly the (3S,4R) isomer with an enantiospecificity higher than >100.[365] Lipase from *Candida rugosa* immobilized on PEI coated supports was the most active hydrolysing 2-phenyl-2-butyroylacetic acid at pH 7 or methyl mandelate.[366]

8.6. Use of PEI to co-immobilize cofactors, conductive molecules and enzymes

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In biosensor design, a good conductivity between enzyme and electrode is a critical requirement for good performance.[367] In this sense the modification of PEI with different molecules and their coimmobilization with the enzyme has been a successful alternative (Figure 13). For example, glucose oxidase coated with PEI was immobilized on polypyrrole film deposited on poly(styrene sulfonate) modified glass, mica and indium-tin-oxide coated glass plates.[368] The immobilization of glucose oxidase on a conductive polypyrrole film permitted to build glucose biosensors. In another paper, PEI was adsorbed on the surface of a screen-printed carbon electrode and ferricyanide ions were adsorbed and confined inside the polymer bed.[369] This was incubated in a $FeSO_4$ solution, to permit Fe^{2+} cations to react with the adsorbed $[Fe(CN)_6]^{3-}$. The reaction led to the formation of Prussian blue crystals that were very active in the electrochemical reduction of hydrogen peroxide. After glucose oxidase was immobilized via ion exchange on the electrode surface, a biosensor for glucose was produced. This sensor reduced the redox activity of many interfering compounds (Figure 13).[369] PEI was modified with ferrocene groups and the modified polymer was adsorbed on multi-wall carbon nanotubes attached carbon cloth.[370] Then, it was crosslinked with glutaraldehyde and glucose oxidase was covalently immobilized onto this electrode.

A step beyond in the design of biosensors was the modification of PEI with ferrocene and NAD^+. This polymer was coimmobilized in an electrode with the enzyme alcohol dehydrogenase to build a reagentless alcohol biosensor.[371] A similar strategy was also used to coimmobilize PEI-ferrocene/NAD^+ polymer and L-lysine 6-dehydrogenase from *Geobacillus stearothermophilus* in a gold sensor to build a reagentless L-lysine sensor. Several layers of enzyme and polymers were used to increase the sensor sensibility. PEI was covalently modified with NAD^+ and 3,4-dihydroxybenzaldehyde.[372] The polymer displayed both electrochemical properties of catechol (useful as electron mediator) and biological activity of NAD moieties when using alcohol dehydrogenase. Just a slight increase in the Michaelis-Menten constant was observed with the polymeric NAD^+. The

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bioelectrochemical properties of this double modified polymer were used to develop a reagentless biosensor using glucose-6-phosphate dehydrogenase. The polymer and the enzyme were retained on the electrode surface by using an ultrafiltration membrane.[372]

Nowadays the use of enzymes that require cofactors (NAD(P)H/NAD(P), ATP, UDP) is one of the aims of biocatalysis, as not only the cofactor regeneration but also its reuse for several reaction cycles may determine the implementation of many processes.[373] The immobilization of the cofactors on polymers is one of the preferred ways to facilitate this reuse, and PEI is one of the polymers used (as shown in the design of biosensors above). Related to biocatalysis, poly(allylamine) was modified with NADH and trapped with several dehydrogenases in hollow-fiber and used to produce methanol from CO_2.[374]

In a very recent paper, López-Gallego and coworkers have shown that phosphorylated cofactors (PLP, FAD^+, and NAD^+) become "reversibly immobilized" by using supports coated with PEI (Figure 20).[375] It is postulated that the cofactors are retained in the polymer in a reversible way by ion exchange, facilitating the cofactor availability for the enzyme, and with very low levels of lixiviation. Two examples were given. In the first one, alcohol dehydrogenase from *Thermus thermophiles* was immobilized on glyoxyl agarose and PEI was coimmobilized on this support. As regenerating enzyme, they employed the formate dehydrogenase from *Candida boidinii*, which failed in becoming immobilized on glyoxyl agarose in an active form. For this reason, the enzyme was adsorbed on PEI treated with 1,4-butanediol diglycidyl ether to get a covalent immobilization of this enzyme on the PEI layer. Then, the combi-biocatalyst was incubated in a NADH solution and the biocatalyst with the adsorbed cofactor was used in the transformation of 2,2,2-trifluoroacetophenone to *S*-(trifluoromethyl)bencylalcohol, just using formic acid to regenerate the cofactor by formate dehydrogenase. The cofactor was re-cycled for 107 h of use (equivalent to 356 operational volumes) without significant NAD+ loses. In another example, racemic methylbencylamine was transformed in

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R-methylbencylamine through S-selective deamination using w-transaminase coimmobilized in glyoxyl agarose with PEI and later incubated with PPL.[375] This paper is of outstanding importance in the design of reactions involving phosphorylated cofactors, and cofactor may be not only recycled but even reused several cycles of reaction. After the cofactor inactivation, if the enzyme remains active, although the authors did not study that, it is very likely that incubation at high ionic strength may release the inactive cofactor and permit the loading of fresh cofactor batch, reusing the coimmobilized enzymes.

6. Conclusions and future trends

From the ideas and results presented in this review, it seems obvious that the impact of PEI in the future may be even greater than the already outstanding one that we are currently experiencing. Its price is very competitive, the handling is simple, and its possibilities of use in biocatalyst design are huge. It is true that some proteins may be readily inactivated by PEI, but they are more the exception than they are the rule.

To take full advantage of these possibilities, a proper activation of the support (having a thick polymeric bed) and a thorough control of the immobilization conditions (to permit the penetration of the enzyme on the bed) are mandatory to have an adsorption involving the maximum percentage of the enzyme surface. This will permit to take full advantage of the use of this polymer, reaching a strong enzyme adsorption and also a higher protection of the enzyme from inactivating agents. Among the challenges that may limit the utilization of PEI in the design of biocatalyst is the possibility of release of the enzyme if the PEI is used to coat the support, or of the enzyme if the enzyme is coated with PEI. This will produce undesired effects and a contamination of the medium. One general solution may be to use enzymes with a surface greatly enriched in anion groups, and if possible, with a reduction in cationic groups. This may be achieve via chemical or genetic modification, and may help to solve this

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problem. Very recently, it has been reported a possibility to prevent PEI desorption from PEI coated immobilized: the chemical immobilization of the PEI in enzymes surfaces activated with bifunctional reagents, like glutaraldehyde. This fully prevents this problem under any condition and may be sued to coimmobilized enzymes or enzymes and cofactors.

Other drawback is also an advantage: the polymer flexibility. This flexibility avoids the generation of strong diffusion problems (that is positive), but also did not introduce rigidity to the enzyme, that will be that way hardly rigidified. This makes that PEI is not valid to immobilize or even intramolecular crosslink enzymes when the objective is increase enzyme rigidity to enhance enzyme stability. However, it may be very adequate when stability is not a problem, because the enzyme is already very stable in the operation conditions (e.g., thermophilic enzymes). Otherwise, enzyme rigidification should be accomplished by other genetic or chemical strategies.

This polymer flexibility also produces a further inconvenience: enzymes that may become inactivated by interactions in partially hidden pockets may be inactivated using PEI coated supports and not standard aminated supports. Branched PEI may limit this effect, if this may be controlled.

Moreover, this polymer is multifunctional, and it may permit partial modifications to tailor the final properties, or to introduce some required groups. This application is underutilized in the current literature and may become one of the greatest advantages of using a multifunctional polymer like PEI.

Some possibilities related to PEI are clearly infra-utilized, for example the use of PEI to produce multi-layers of enzymes on surfaces where the specific area is small. This may permit to dilute the enzyme with a random coil polymer (reducing mass transfer limitations) and to introduce high amounts of protein per surface unit. Even if some substrate diffusion limitations occur, this may not be detrimental for certain applications, e.g., it may enlarge the range of concentrations where a biosensor may increase the response when the concentration of the analyte is high. Some likely advantages of

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PEI still remain unexplored. One point that (surprisingly) has not been studied at all is the strong buffering properties of PEI. We have not found any application of this property, but at first glance it may be enough to reduce or even fully prevent some pH gradients formed in enzyme reactions releasing basic or acids compounds, and it may also improve enzyme stability in titration processes by preventing enzyme exposition to drastic pH values.

Some others features are used, but have been very recently described and a wider diffusion of these characteristic is expected in the near future. Stabilization of multimeric enzymes, even immobilization of multi-enzymatic complexes is another application that should have more impact due to the simplicity of the protocols and efficiency of the treatments. Similarly, the physical crosslinking of physically immobilized enzyme molecules and the generation of enzyme nanoenvironments opens up new possibilities to solve some problems of very often used biocatalysts, approaching these biocatalysts to the industrial implementation for new processes. Prevention of enzyme inactivation due to interaction with hydrophobic interfaces (e.g., gas bubbles, drops of insoluble solvents) will have a more relevant role when the use of non-porous magnetic nanoparticles to immobilize proteins has a wider application.

However, the combination of two ideas very recently presented may be the main future application of PEI in biocatalysis. This is the involvement of PEI in cascade enzymatic reactions involving phosphorylated cofactors and several enzymes (Figure 21). Many enzymes involved in these reactions are multimeric ones that may be stabilized just via ion exchange in a polymeric bead involving all enzyme subunits, but in some cases they cannot be stabilized by multipoint covalent attachment for different reasons. Thus, among the enzymes involved in the cascade reaction, enzymes that are more stable or that can be significantly stabilized via multipoint covalent attachment may be immobilized following optimal protocols to improve at the maximum the enzyme properties. Later, they can be coated with PEI, which may present many positive effects on the immobilized enzyme

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stability. If desired, it is possible to covalently attach PEI to the enzyme or to the support to prevent PEI desorption. Next, the second enzyme may be ionically exchanged on this polymer-coated enzyme, and finally, the combi-catalyst-PEI can be incubated in the solutions of the cofactors. That way, the cofactor may be reused for several cycles, and the least stable enzyme discarded after inactivation enabling the reuse of the most stable enzyme. The possibility of modifying PEI with different reagents may permit to facilitate electron transmission, or also enable the second enzyme to be immobilized via another reversible mechanism (e.g., thiol exchange). Genetic or chemical enrichment of the enzyme surface on anionic groups can make stronger second enzyme ion exchange if necessary. Combi-CLEAs of different enzymes and PEI may be incubated in cofactor solutions with similar objectives, although in this case the reuse of the most stable enzyme will not be possible.

Thus, the use of PEI in biocatalysis may be considered that still is in the first steps even though it has been produced for a long time. The road ahead may lead to solutions for biocatalysts design problems that may be solely in our imagination, or even situations which we have not even dared to dream yet.

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Figure legends

Scheme 1. Standard synthetic route to produce branched-PEI (BPEI) via ring-opening polymerization of unsubstituted ethylenimine.

Scheme 2. Alternative synthetic route to produce branched PEI (BPEI) via ring-opening isomerization polymerization of substituted 2-oxazolines.

Scheme 3. Synthesis of Lineal PEI (LPEI) via ring opening polymerization of 2-ethyl-2-oxazoline followed by hydrolysis.

Figure 1.Reversible immobilization of enzyme via ion exchange. The support may be reused after enzyme inactivation by incubation under more drastic conditions than operation ones.

Figure 2. Stabilization of multimeric enzymes via multi-subunit immobilization and physical crosslinking with PEI. Multimeric enzymes subunits may dissociate during operation (e.g., by dilution). Physical crosslinking of the no immobilized subunits with subunits immobilized on the support may prevent this phenomenon.

Figure 3. Stabilization of multimeric enzymes via multi-subunit crosslinking with PEI and further immobilization on anion exchangers. The multimeric enzymes may be coated with PEI to prevent subunit dissociation. This composite will be very rich in cationic groups and can be strongly immobilized on anionic supports, even if the unmodified enzyme cannot become immobilized on these supports.

Figure 4. Generation of nano-enviroments around enzyme surface by coating with PEI. The cationic nature of PEI may form a hydrophilic shell surrounding the enzyme molecule, forming polymeric salts surrounding the enzyme molecule.

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Figure 5. Change of enzyme properties by physical coating with PEI. The interaction of an enzyme with a cationic polymer may produce difficulties to the movement of the enzyme or induce conformational changes. These changes should be reversible if the PEI is desorbed.

Figure 6. Stabilization of enzyme by coating with PEI versus organic solvents: generation of hydrophilic nano-environments to generate solvent partition.

Figure 7. Multilayer immobilization using PEI as glue. After coating the support with a layer of the enzyme, this is activated by coating with PEI and a new layer of enzyme is immobilized. This may be performed continuously until the increment of activity is not compensate by the immobilization of new layers of enzyme (diffusional problems to the iner layers will increase with the production of additional layers of PEI/enzyme).

Figure 8. Effect of PEI concentration in the final thickness of the polymeric bed. Using a high concentration of PEI, the support is rapidly coated by many PEI molecules and do not permit to maximize enzyme-support interactions, enabling the formation of thick polymeric beds. Using low concentration of PEI, each PEI molecule may maximize their interactions with the supports because no other PEI molecule will be there avoiding this, forming a thin polymeric bed.

Figure 9. Immobilization of enzyme sin complex reactors using PEI activation of the support walls. Some bioreactors require a long time to become fixed. In these cases it may be convenient to use reversible strategies of immobilization to avoid the necessity to prepare the reactor anew.

Figure 10. Reuse of PEI activated supports after enzyme inactivation: formation of enzyme-support composites with maximized enzyme-support interactions during enzyme inactivation that may complicate to achieve a fully clean support for immobilizing a new bach of protein

Figure 11. Immobilization of enzymes in PEI coated nano-particles. The enzyme is immobilized on the surface of the support, that way it is able to interact with large structures. This makes that a

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properly oriented enzyme may be active after immobilization even versus solid substrate but also may become inactivated by interaction with hydrophobic surfaces, like gas bubbles.

Figure 12. Buffering effect of PEI: reduction of the pH gradients. In some enzyme reactions, e.g hydrolysis of esters at alkaline pH values, a pH gradient may be produced inside the biocatalyst particle if the enzyme activity is high- enough. This pH drop inside the particle in many instances may reduce the enzyme activity or stability. The presence of PEI may be expected to reduce this internal pH gradient due to their high buffering capacity.

Figure 13. Design of an auto-sufficient glucose biosensor using the multifunctional nature of PEI. Mediators and GOS are immobilized on the PEI bed to get a glucose biosensor that work without the addition of external mediators**.**

Figure 14. PEI coated cells to immobilize enzymes on the cell wall, and their further coimmobilization. This way, enzymes and cells may act in a synergic way.

Figure 15. Production of PEI capsules to immobilize enzymes.

Figure 16. Use of PEI as enzyme-enzyme glue to co-immobilize two enzymes enabling the reuse of the most stable one. The first enzyme may be immobilized using optimal protocol to stabilize it, then it is coated with PEI and a second enzyme and less stable may be immobilized on the first activated enzyme. After inactivation of the second enzyme, this may be desorbed and the most stable one reused after immobilizing a new batch of the second enzyme.

Figure 17. Use of PEI in the preparation of crosslinked enzyme aggregates (CLEAs) of poor Lys enzymes. Crosslinking may be difficutl if the enzyme surface is poor in amino groups. Modification of the enzyme with PEI provide this amio groups and simplify the crosslinking**.**

Figure 18. Physical crosslink with PEI of enzyme molecules physically adsorbed on hydrophobic supports. Lipases immobilized on hydrophobic supports present very good performance but they can be released at high temperature, inprenseec of organic solvents or detergents, even certain substrates or

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product with detergent properties. The coating of the immobilized enzymes with PEI permits the physical intermolecular crosslinking and that way the prevention of enzyme release.

Figure 19. Stabilization of lipase open form induced by detergents using PEI coating. Detergents shift the conformational equilibrium of lipases towards the open form, but after washing it, the enzyme recovers the equilibrium. The coting of the lipases ion presence of detergent has proved to be useful to keep this detergent induced open form of the lipase.

Figure 20. Coating of co-immobilized enzymes with PEI permits the coimmobilization of phosphorylated cofactors.

Figure 21. Use of PEI to coimmobilize several enzymes and their phosphorylated cofactors.

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1649 **Table 1. Examples of enzyme immobilized on PEI coated supports.**

Enzyme	Support	Application	Reference
Nucleotidase	Polystyrene tube	Determination of hypoxanthine	82
Nuclease P1	weak base anion resin	Hydrolysis of DNA	83
Monoamine oxidase	polypropylene membrane	Oxidation of Tyramine	84
Alkaline phosphatase	Plate-shaped ceullose	Hydrolysis of *p*-nitrophenyl phosphate	85
Thermophilic β-galactosidase	Glyoxyl-agarose beads	Hydrolysis of o-nitrophenyl-β-D-galactopyranoside	86
Mesophilic β-galactosidase	Glyoxyl-agarose beads	Hydrolysis of o-nitrophenyl-β-D-galactopyranoside	87
Mesophilic β-galactosidase	Nanofiltration membrane	Production of galactose polymers	88
Mesophilic β-galactosidase	Quartz crystal	Hydrolysis of o-nitrophenyl-β-D-galactopyranoside	89
Mesophilic β-galactosidase	Polymeric membrane	Production of galactose polymers	90
β-glucosidase	Cellulose beads	Wine-making and fruit-juice processing	91
β-Glucosidase	Glyoxyl-agarose beads	Hydrolysis of cellobiose	92
α-Galactosidase	Glyoxyl-agarose beads	Hydrolysis p-Nitrophenyl-a-D-galactopyranoside	93
β-xylosidase	Glyoxyl-agarose beads	Hydrolysis of xylooligosaccharides and production of xylooligosaccharides (X2-X6)	94
Haloalkane dehalogenase	γ-alumina beads	Hydrolysis of haloalkane	95
Glucoamylase	Glyoxyl-agarose beads	Hydrolysis of maltose or starch	96
Invertase	Poly(GMA–MMA) beads	Hydrolysis of sucrose	97
Glutaryl Acylase	Epoxy Sepbeads	Hydrolysis of glutaryl-7-ACA	98
Cephalosporin C acylase	Epoxy-LX1000-EPC4	Hydrolysis of cephalosporin C	99
Tyrosinase	Poly(hydroxyethyl methacrylate-co-glycidyl methacrylate) membranes	Transformation of L-Tyr to tyrosinaseo-benzoquinine	100
Tyrosinase	Agar particles, blocks and egg shell	Transformation of L-tyrosine to L-3, 4 dihydroxyphenylalanine	101
Gutamate oxidase	Platinum electrode	Detection of Glu	102

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Formate dehydrogenase	Glyoxyl-agarose beads	Oxidation of formic acid, regeneration of NADH	103,104
Penicillin acylase	SiO_2 beads	Hydrolysis of penicillin G	105,106
Alcohol oxidases	Glyoxyl-agarose beads	Ethanol oxidation	107
Glucose oxidase	Screen-printed carbon electrodes	Glucose detection	108
Thermophilic alcohol dehydrogenase	Glyoxyl-agarose beads	Reduction of acetophenone to (S)-(-)-1-phenylethanol	109
Peroxidase	Electrodes with zirconium alcoxide film	Detection of acetaminophen	110
Urease	Egg shell	Detection of urea	111
Laccase	Chitosan	Oxidation of syringaldazine	112
Pectinase	Pulp fiber	Treatment of whitewater from papermaking	113
Cellobiose dehydrogenase	Graphite Electrodes	Cellobiose quantification	114
Pepsin	Epoxy Sepabeads	Hydrolysis of immunoglobulins to produce F(ab')2 fragments	115
Polygalacturonase	Glyoxyl-agarose beads	Juice clarification	116

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1653 **Table 2. Examples of enzyme immobilized on PEI coated supports and treated with**

1654 **glutaraldehyde.**

Enzyme	Support	Application	Reference
Glucoamylase	Porous glass beads	Hydrolysis of starch	129
Glucose oxidase and catalase	Cotton cloth	Production of spray-dried, sugar-free egg powder	130
Alkaline phosphatase	Silk fabric	Hydrolysis of disodium 4-nitorophenyl phosphate	131
Invertase	Rice husk	Hydrolysis of sucrose	132
Urease	Glass-sealed metal microelectrode	Urea detection	133
β-galactosidase	cotton cloth	Galacto-oligosaccharides production	134, 135
β-galactosidase	Polyether sulfone membrane	Galacto-oligosaccharides production	136
β-galactosidase	Poly (acrylonitrile-	Hydrolysis of lactose	137

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	co-methyl methacrylate) poly (AN-co-MMA) Nanofibers		
β-galactosidase	Flat-sheet ultrafiltration membrane	Galacto-oligosaccharides production	138
D-amino acid oxidase	Glyoxyl agarose beads	Oxidative deamination of Ala and cephalosporin C	139
Lipase	Cotton cloth	Esterification of butanol and butyric acid	140
Lipase	Cotton terry cloth fibrils	Hydrolysis of p-nitrophenyl propionate	141
Lipase	Woolen cloth	Hydrolysis of tributyrin emulsion	142
Lipase	Polyurethane foam	Production of geranyl propionate	143
Urease	Cotton cloth	Hydrolysis of urea	144

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1656 **Table 3. Examples of enzyme immobilized on PEI-glutaraldehyde pre-activated supports.**

Enzyme	Support	Application	Reference
trypsin	Silica gel	Hydrolysis of N-cr-benzoyl-L-arginine ethyl ester and N-o-tosyl-L-arginine methyl ester	149
Lactate dehydrogenase	Silica gel	Pyruvate reduction	149
Chymotrypsin	Silica gel	Hydrolysis of N-o-acetyl-L-tyrosine ethyl ester and N-cx-benzoyl-L-tyrosine ethyl ester	149
Glucose oxidase	Nylon	Oxidation of glucose	150
Glucose oxidase	Light-addressable potentiometric sensors	Detection of glucose	151
Chymotrypsin	Light-addressable potentiometric sensors	Detection of peptides	151
Urease	Stain etched porous silico	Detection of urea	152
Urease	Polymethylglutamate membrane	Detection of urea	153
Amyloglucosidase and α-amylase	Different inorganic supports	Hydrolysis of maltose, maltooligosaccharides and soluble starch	154
Amyloglucosidase and α-amylase	Different inorganic supports	Hydrolysis of glycogen	155
Glucose oxidase, malate dehydrogenase	Screen-printed electrodes	Following glucose and malic acid in wine production	156
Amino acid oxidase, protease	Rodinised carbon electrode	Protein detection	157
Glucoamylase	Cotton cloth	Hydrolysis of starch	158
Protease C	Different cloth fibers	Antimicrobial activity	159
Glutaryl acylase	Epoxy SepabedS	Hydrolysis of glutararyl-7-ACA	160
Penicillin G acylase	SiO2 beads	Hydrolysis of penicillin G	161
Lysozyme and trypsin	Stainless steel	Generation of anti-biofilm surfaces	162
Peroxidase	Electrospun microfibrous membranes	Degradation of bisphenol A	163
Peroxidase	Aluminum oxide	Oxidation of *o*-phenylenediamine	164
Trypsin	Aluminum oxide	Hydrolysis of *N*-α-benzoyl-dl-arginine-*p*-nitroanilide	164
Invertase	Polyurethane, plast-film	Hydrolysis of sucrose	165

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	and ferromagnetic Dacron		
β-D-galactosidase	Agar disks	Hydrolysis p-Nitrophenyl-a-D-galactopyranoside	166
Lipase	Polyurethane foam	Production of lauryl laurate	167
Lipase	Woolen fabrics	Stain removal	168
Alcohol oxidase	Electrospun fibers	Determination of ethanol in saliva	169
Dextransucrase	Fe3+-cross-linked alginate/carboxymethyl cellulose beads	Dextran synthesis	170
Ppyruvate kinase and L-lactic dehydrogenase	poly(N-isopropylacrylamide) microgel	Transformation of phosphoenol pyruvate on pyruvic acid and ATP and this on lactate with NADH consumption	171

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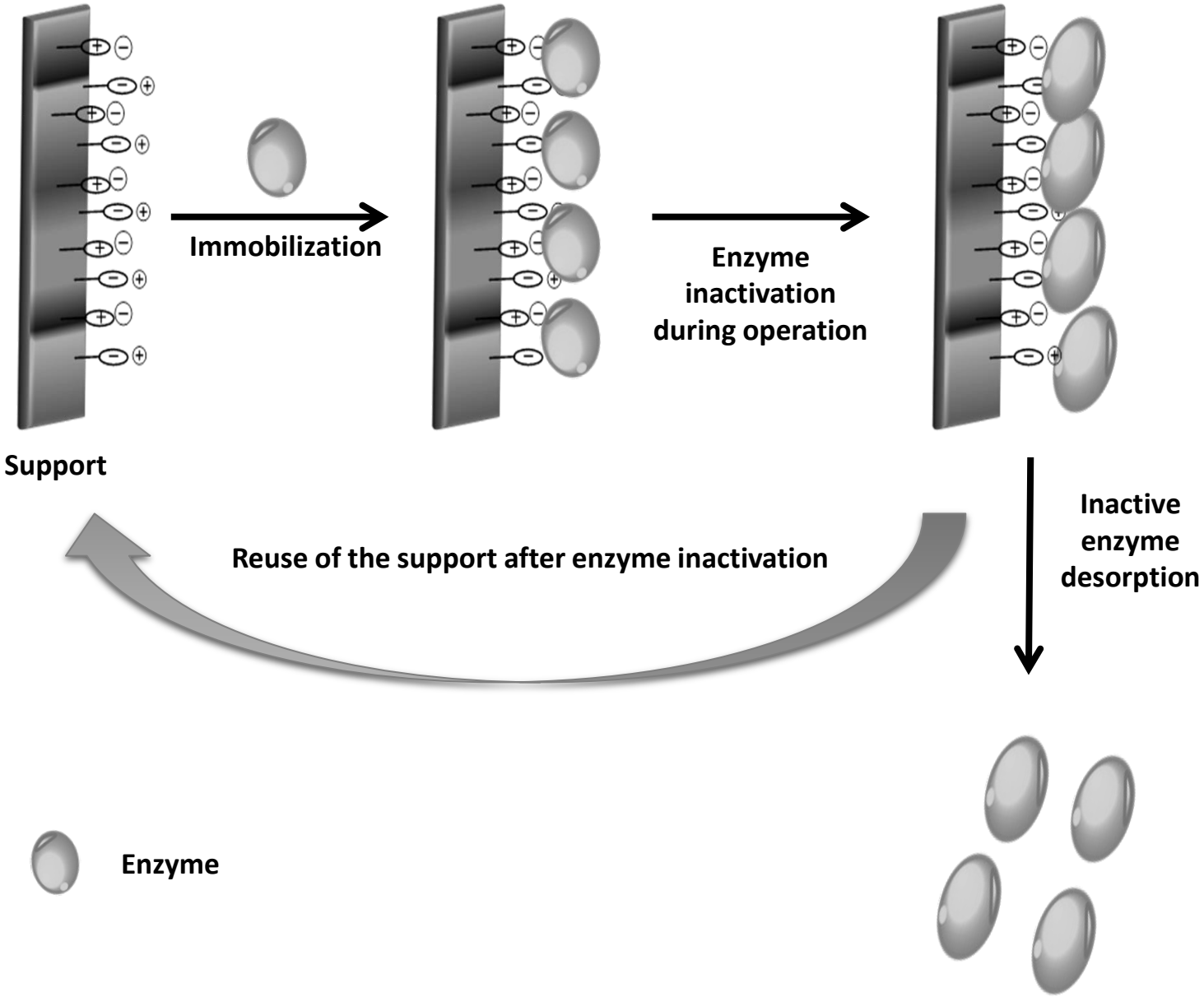
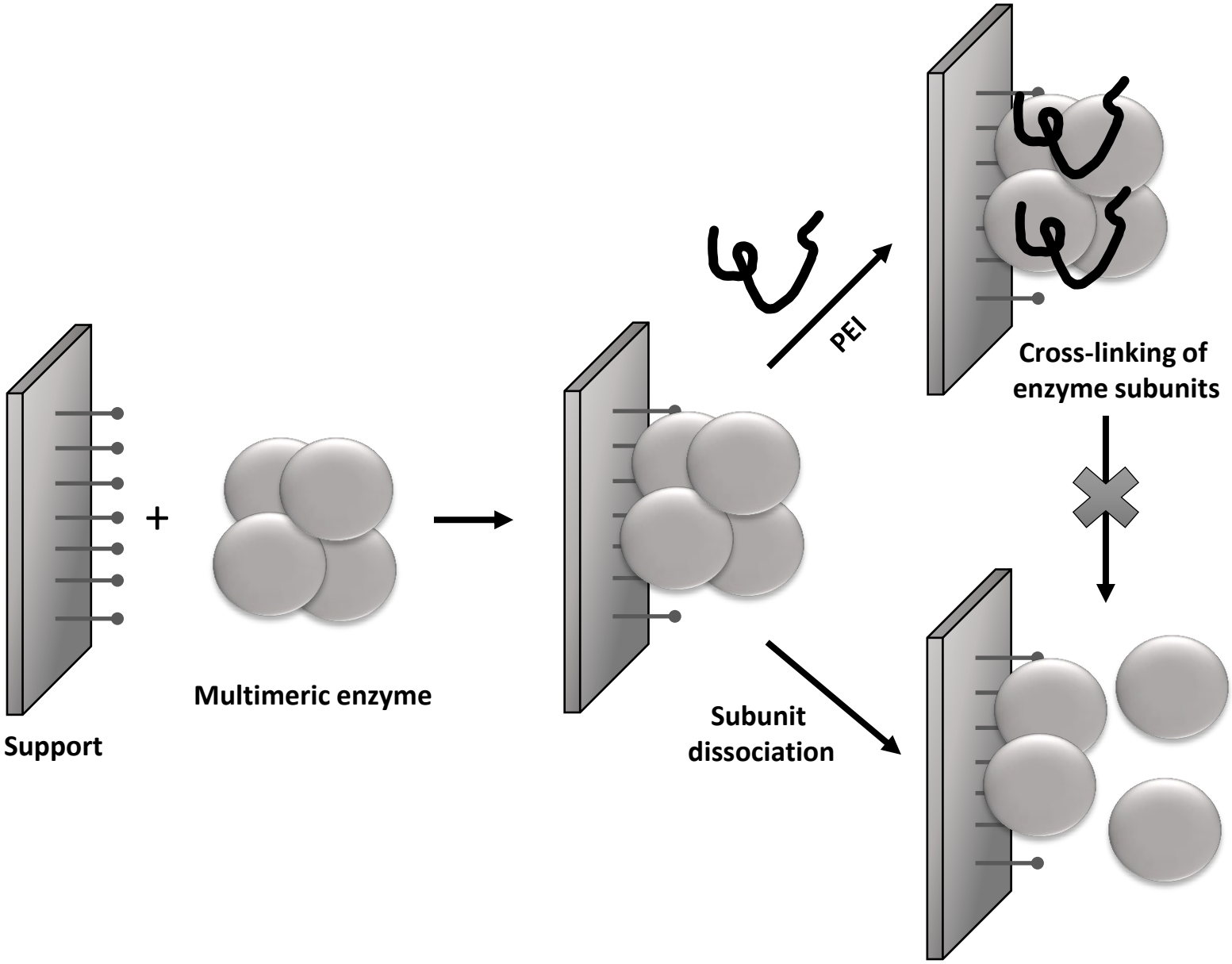


Figure 1



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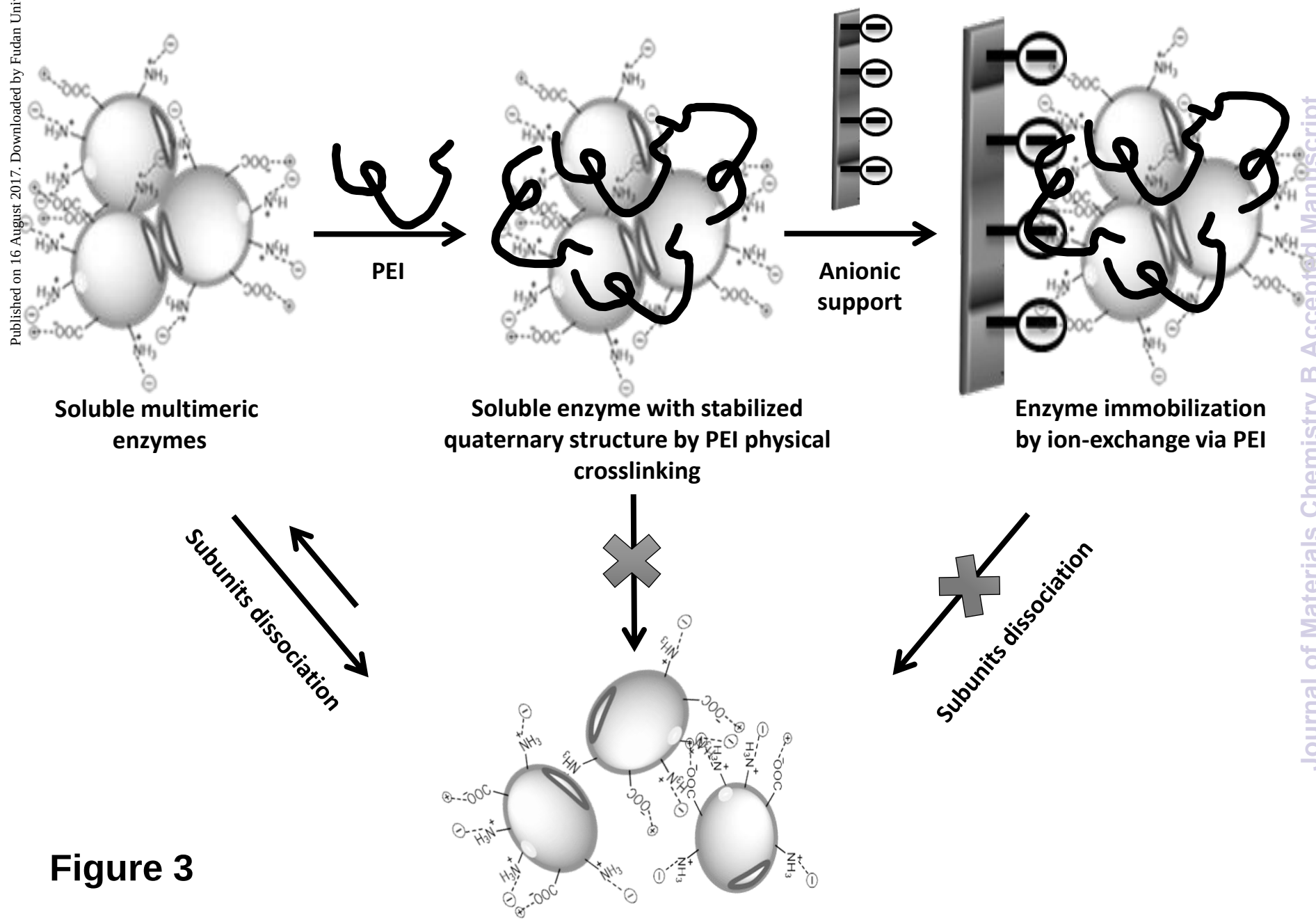
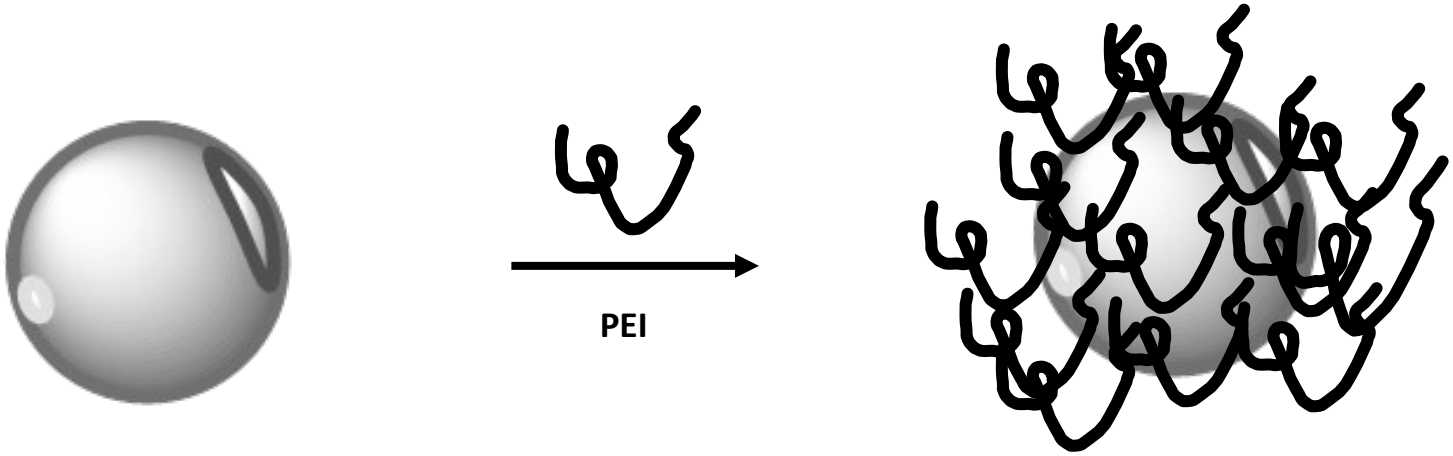


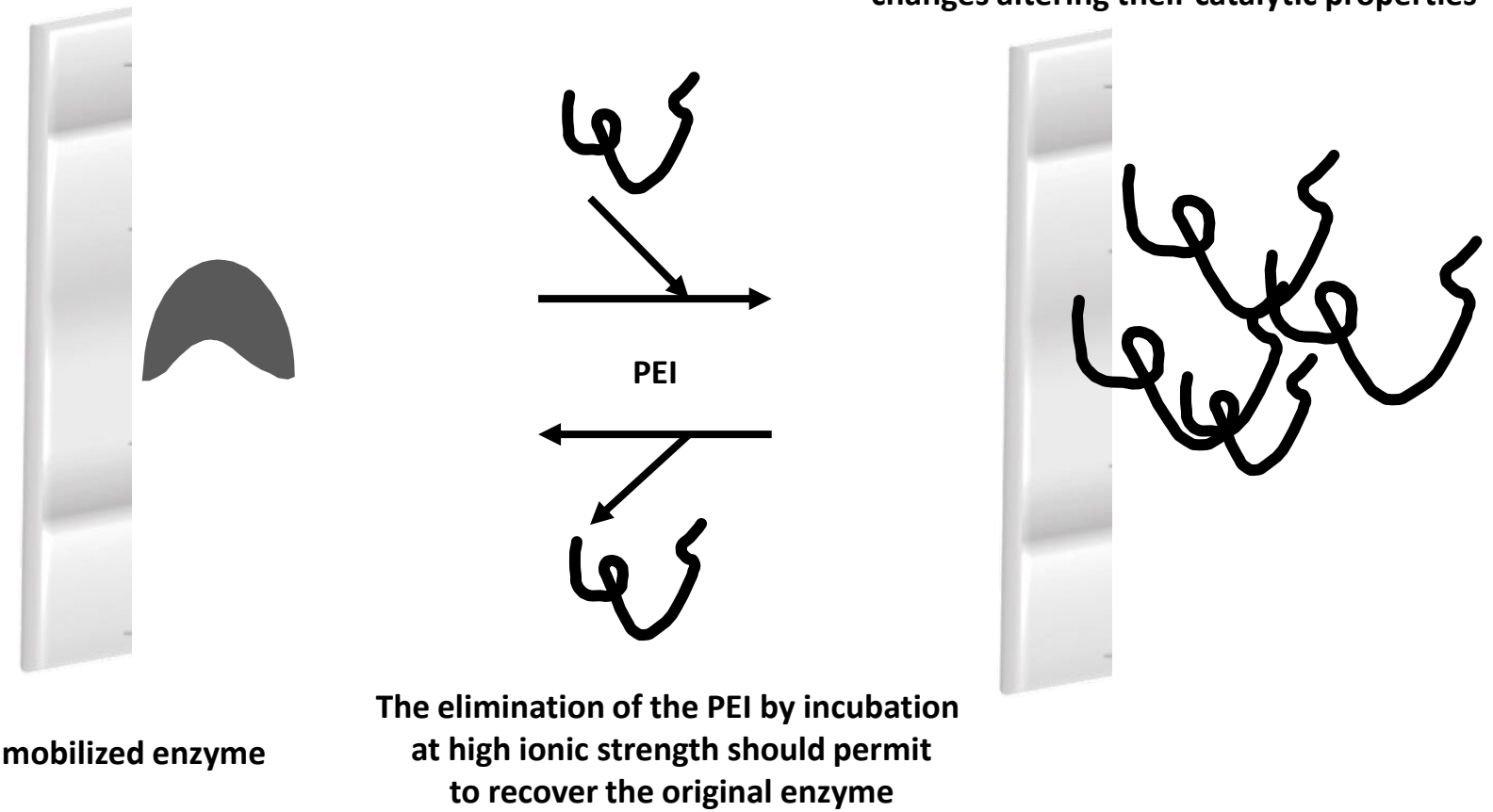
Figure 3



Enzyme in the reaction medium

The enzyme environment becomes the cationic and highly hydrophilic PEI

Figure 4



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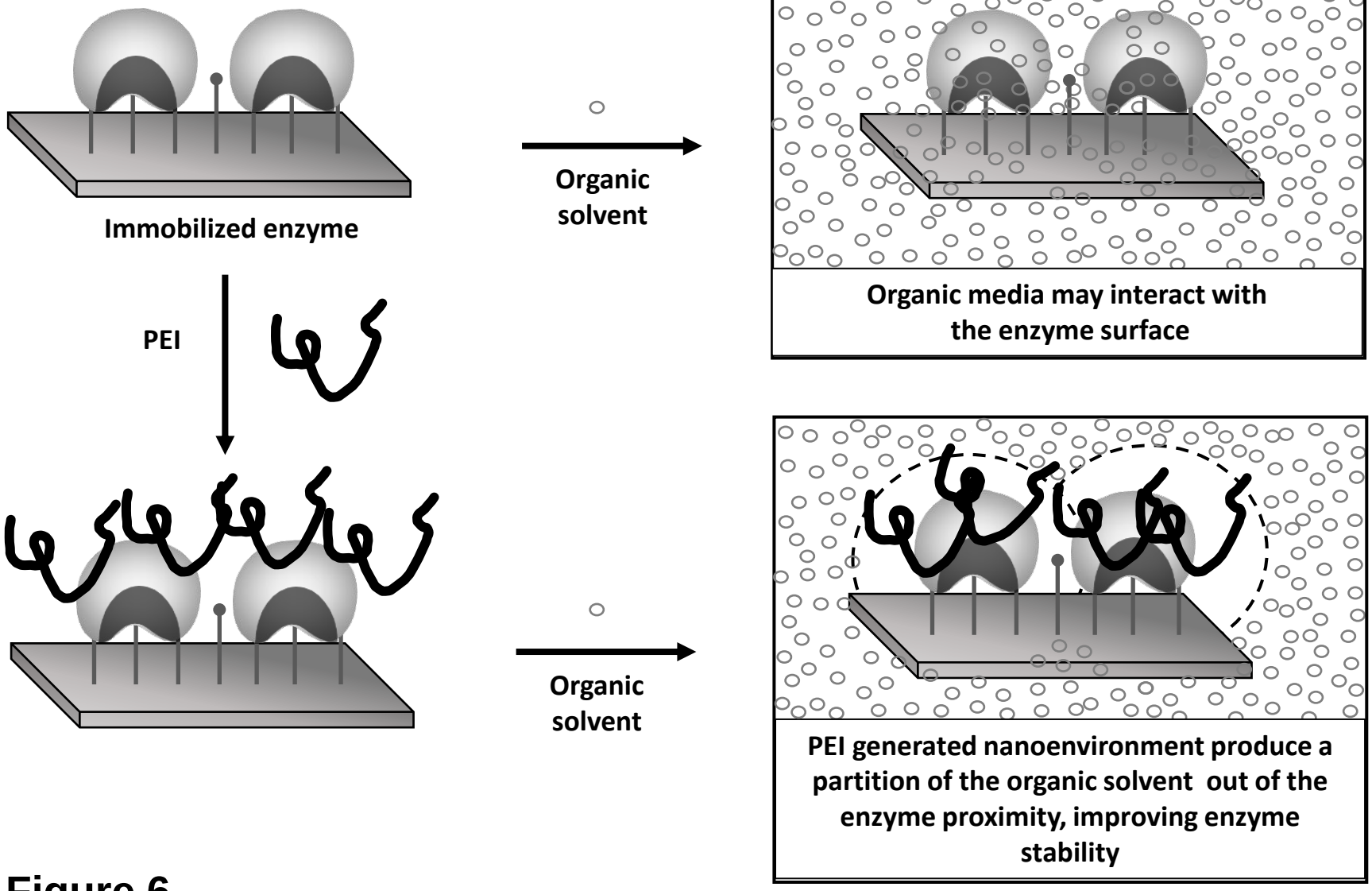

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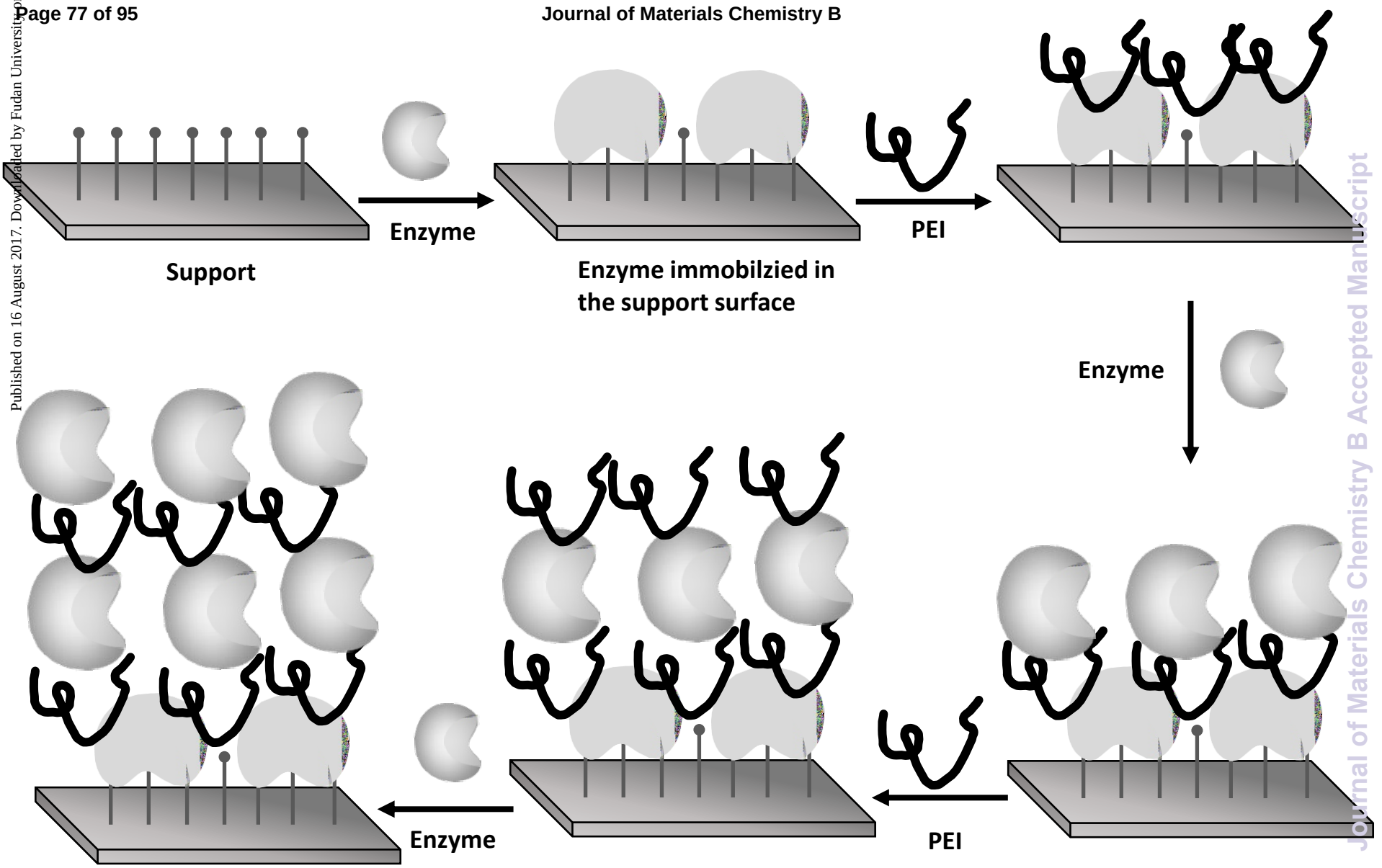


Multilayer immobilization using PEI as glue to increase loading capacity

Figure 7

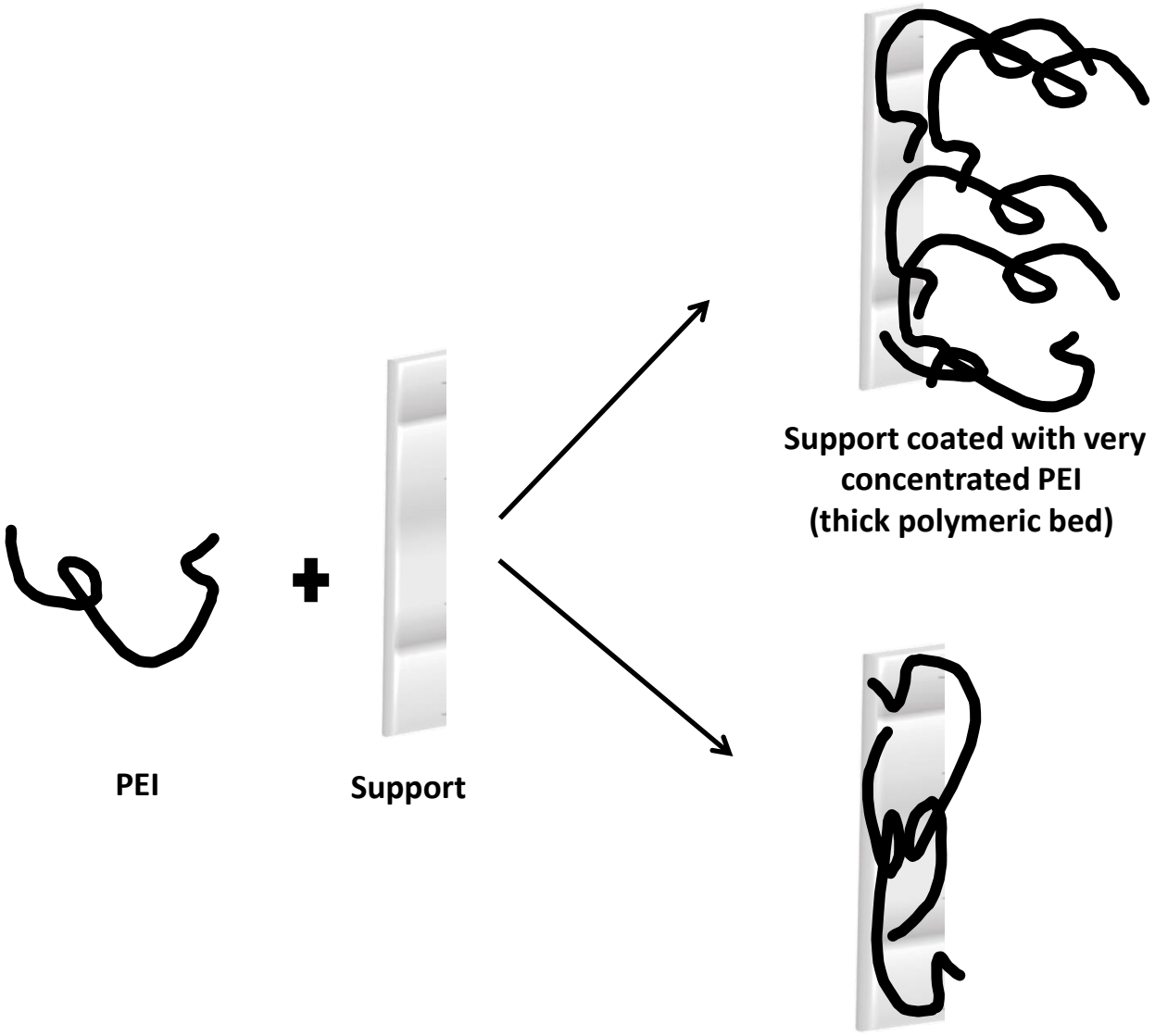
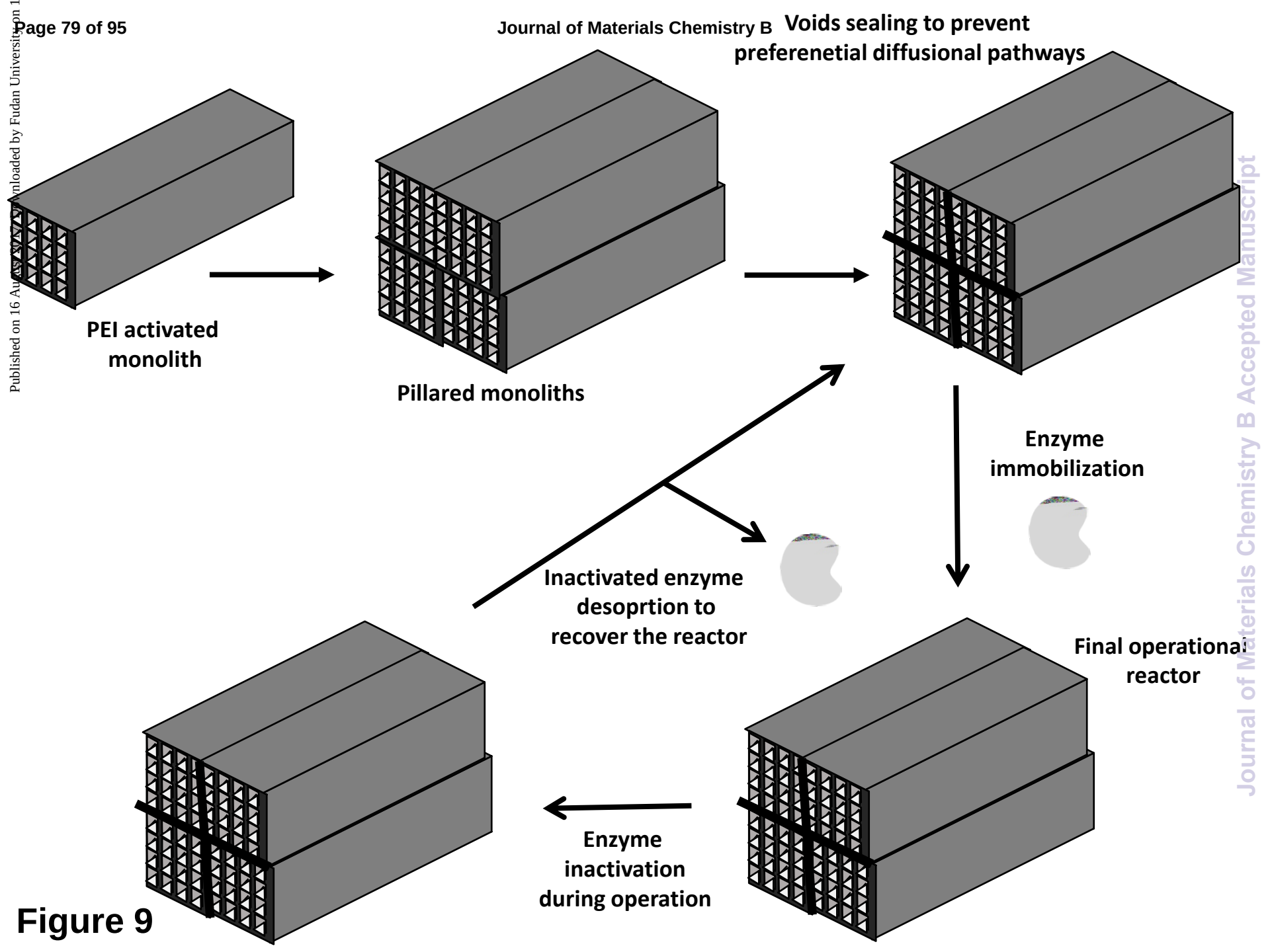


Figure 8



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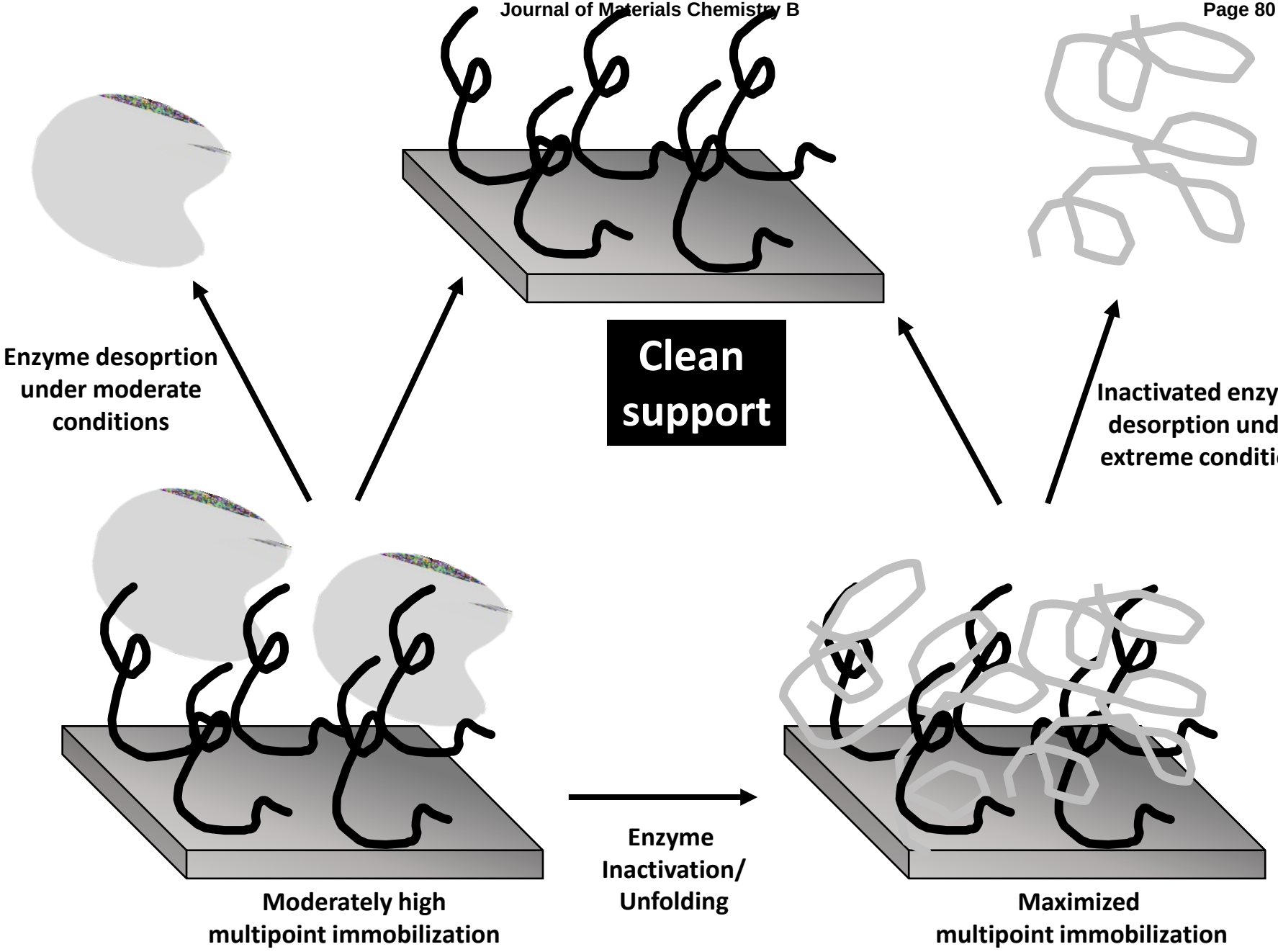


Figure 10

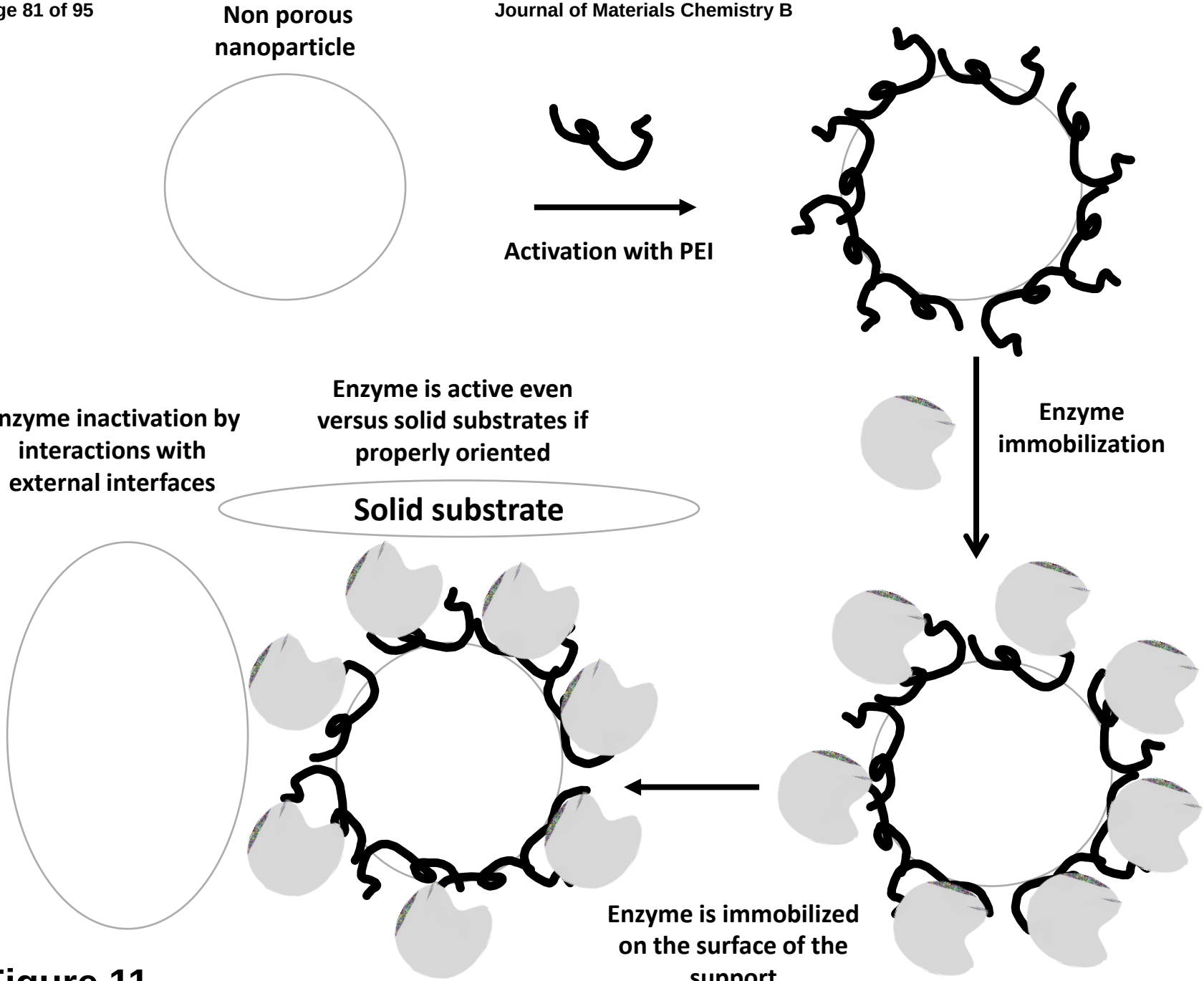


Figure 11

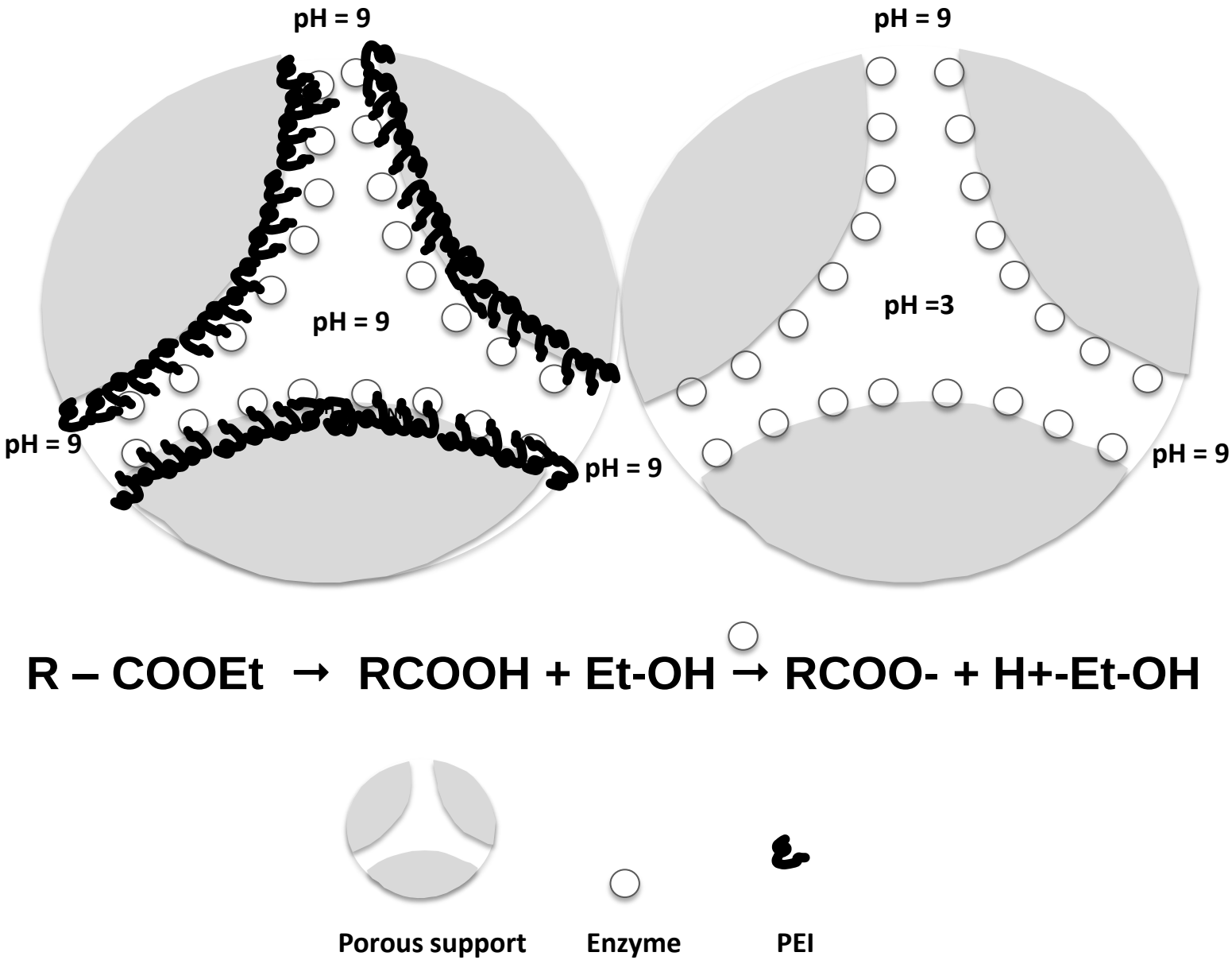
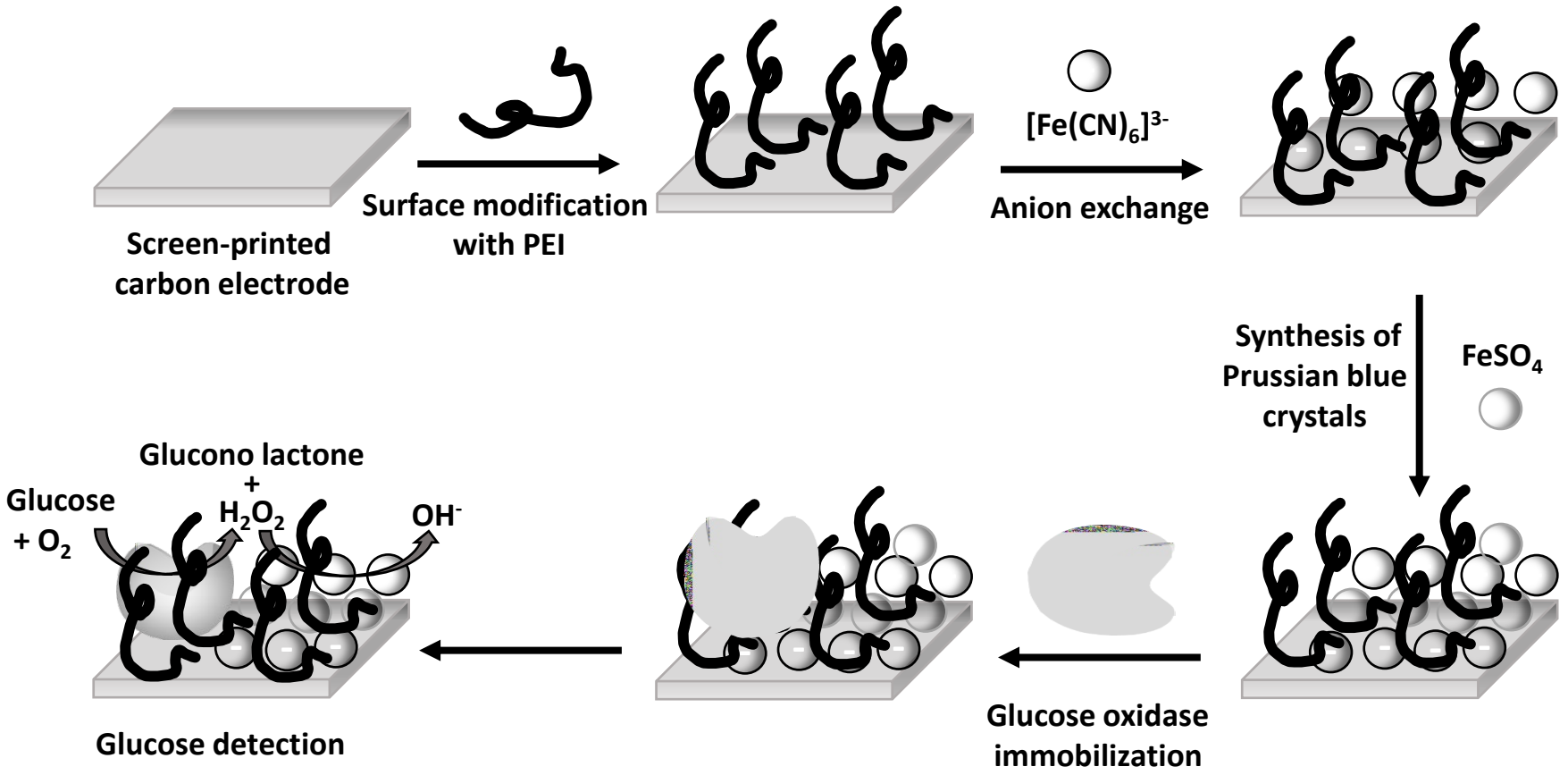
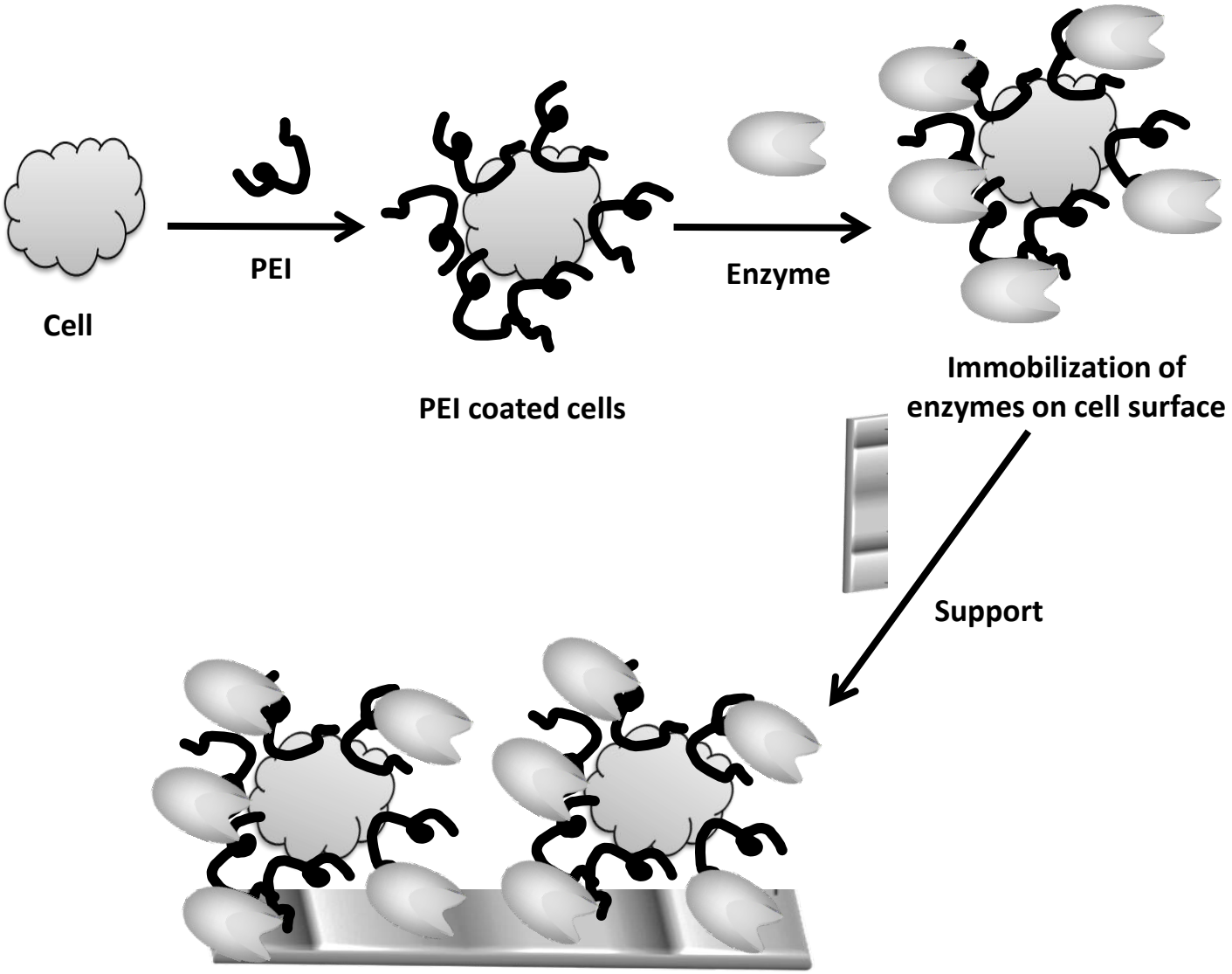


Figure 12



Coimmobilization of GOx and mediators to get a biosensor that not require addition of further compounds to work

Figure 13



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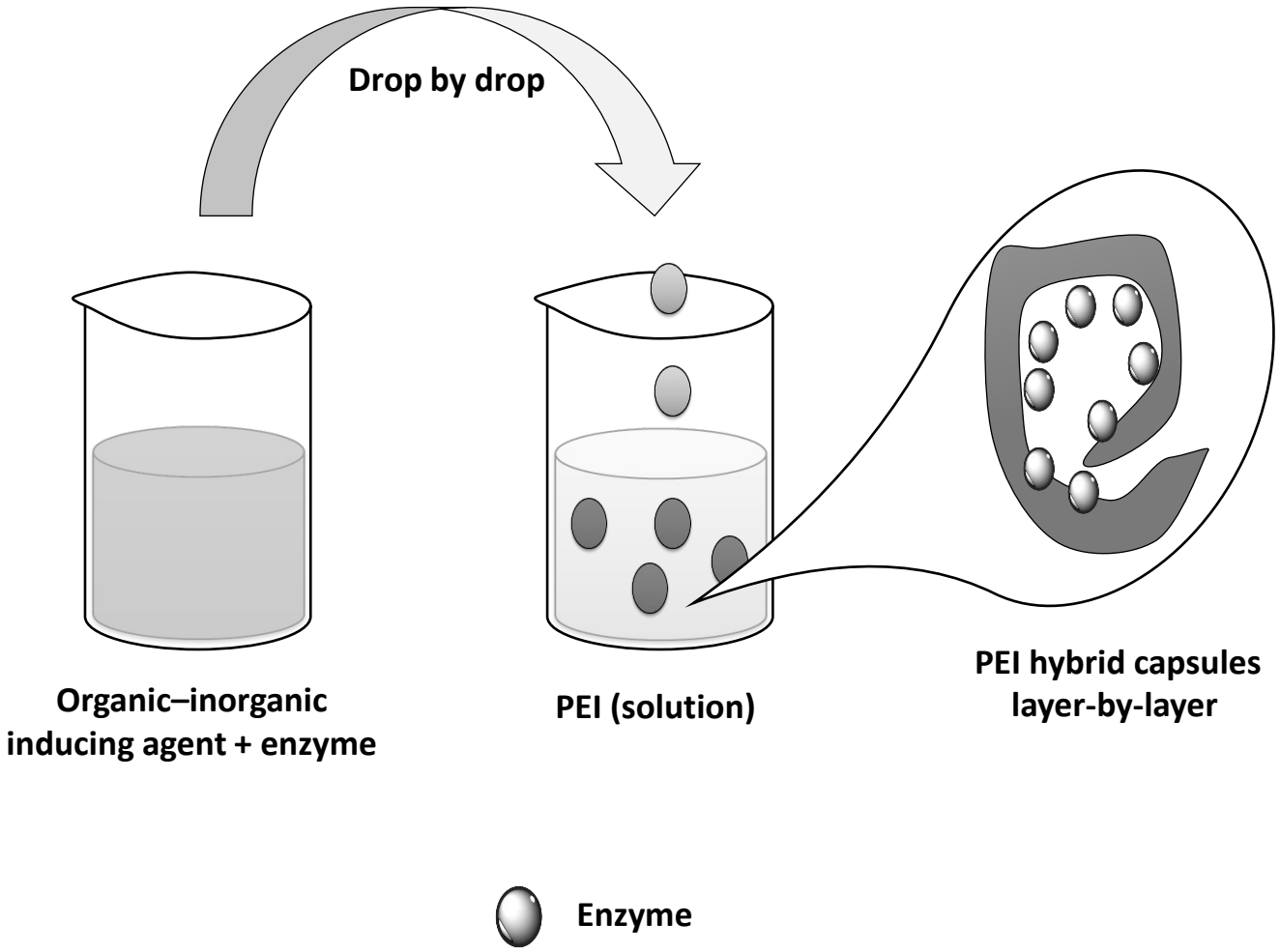
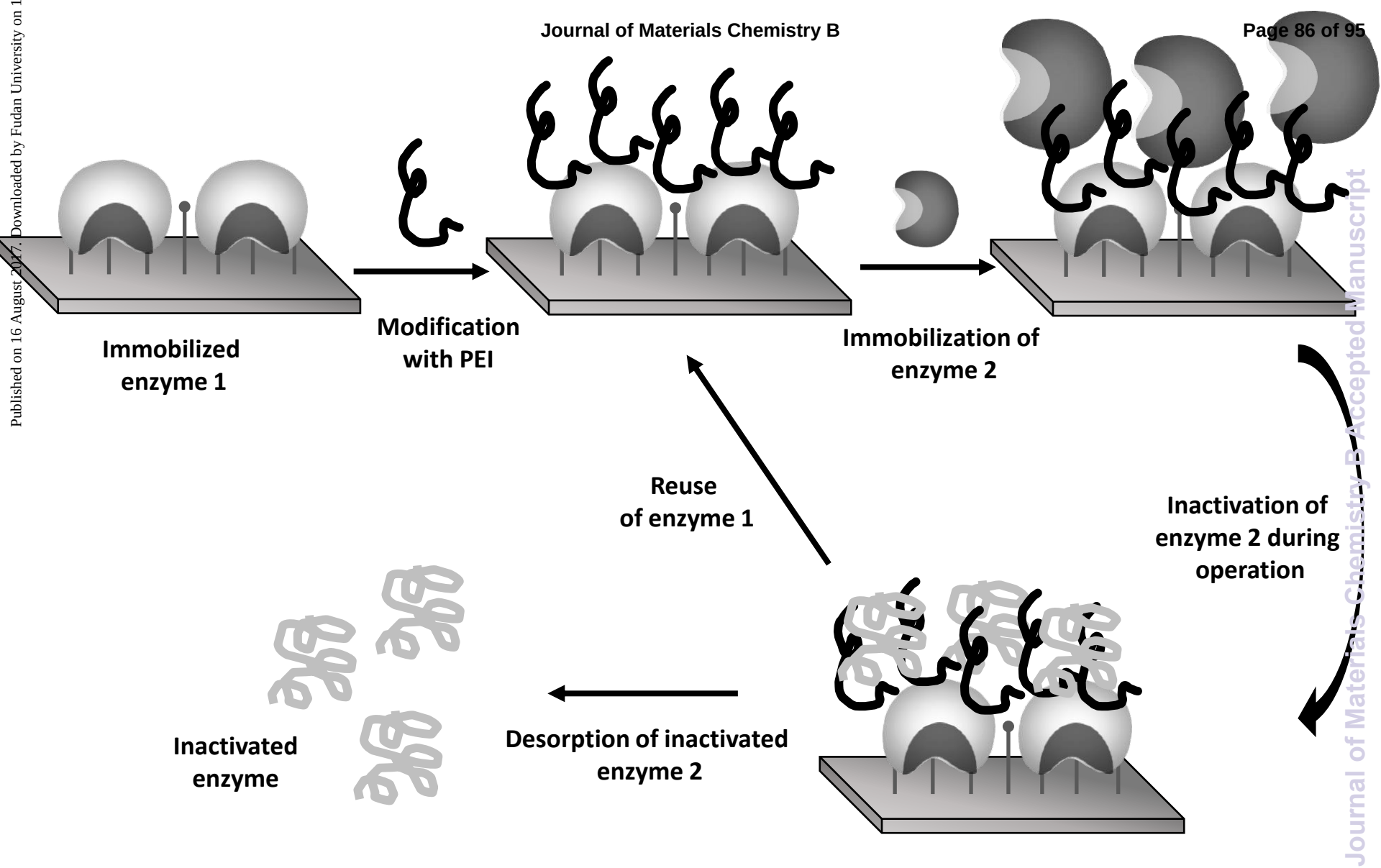


Figure 15



Coimmobilization of two enzymes to reuse the most stable one after inactivation of the less stable enzyme

Figure 16

Journal of Materials Chemistry B

USE OF PEI TO GET STABLE CLEAs

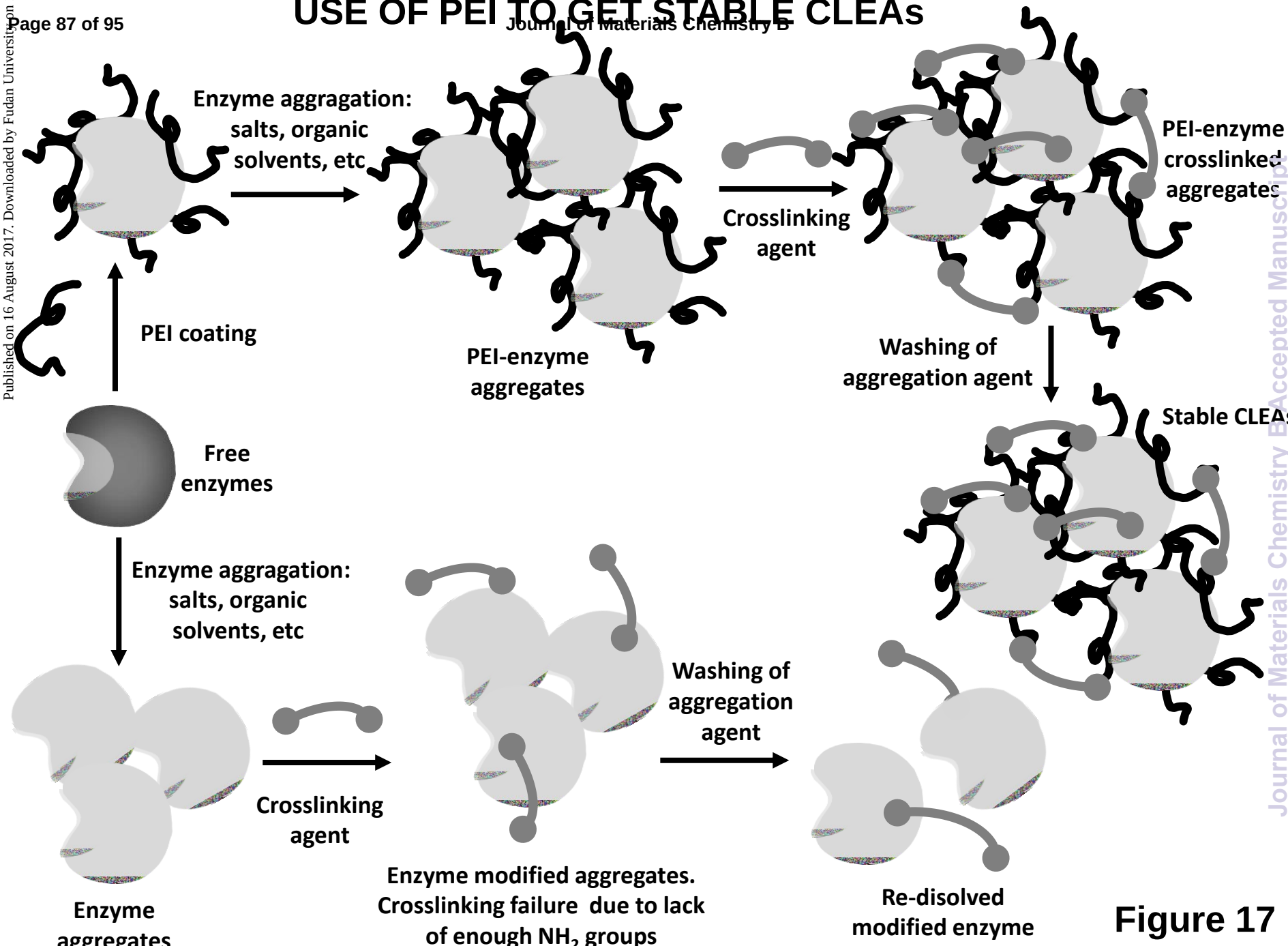


Figure 17

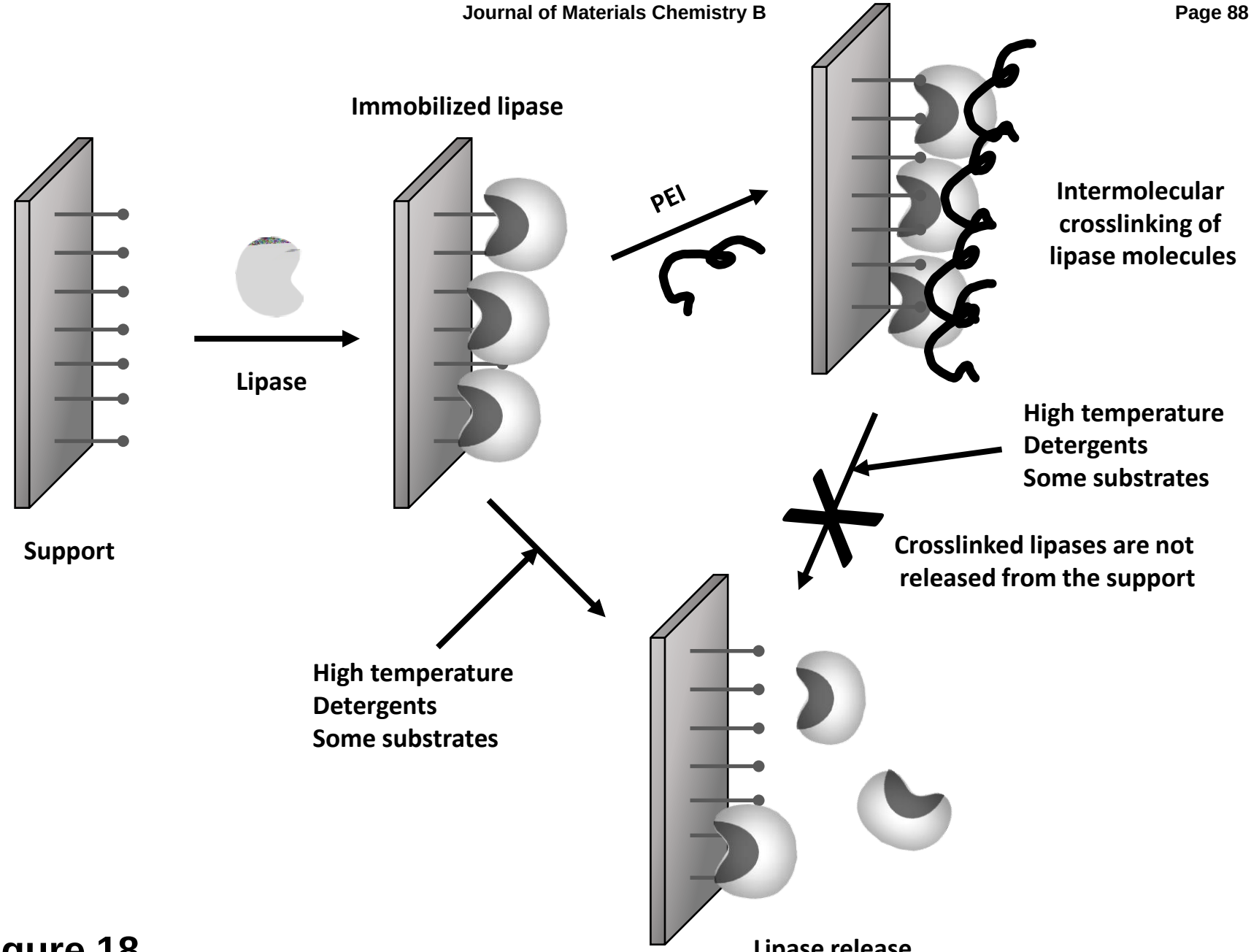


Figure 18

Bioimpriting of the open form of lipases induced by detergent using PEI

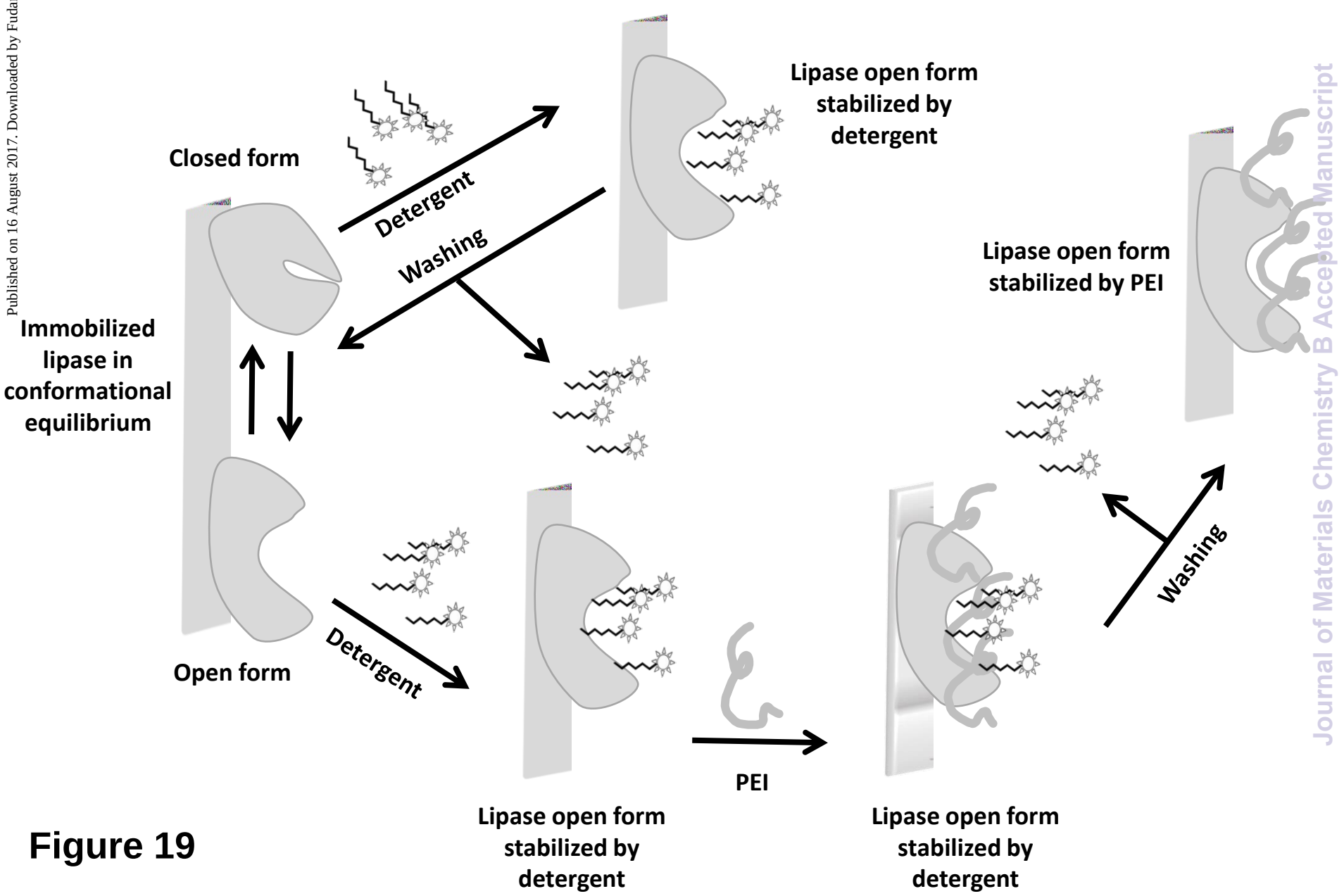


Figure 19

Active cofactor immobilization on immobilized enzymes: recycling and reuse of cofactors

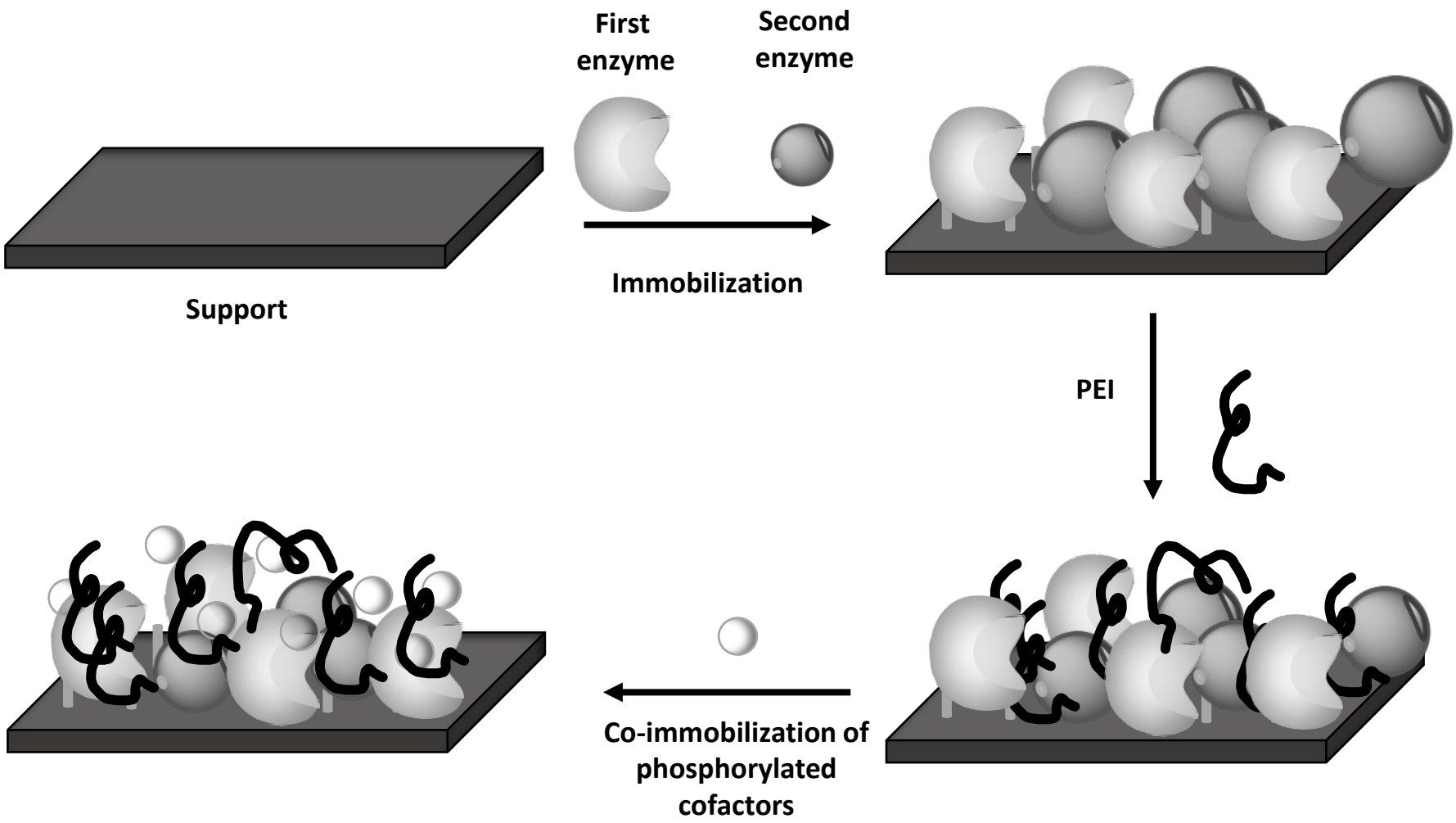


Figure 20

Use of PEI to coimmobilize two enzymes and a fosforilated cofactor: Reuse of the most stable enzyme and reuse of the cofactor

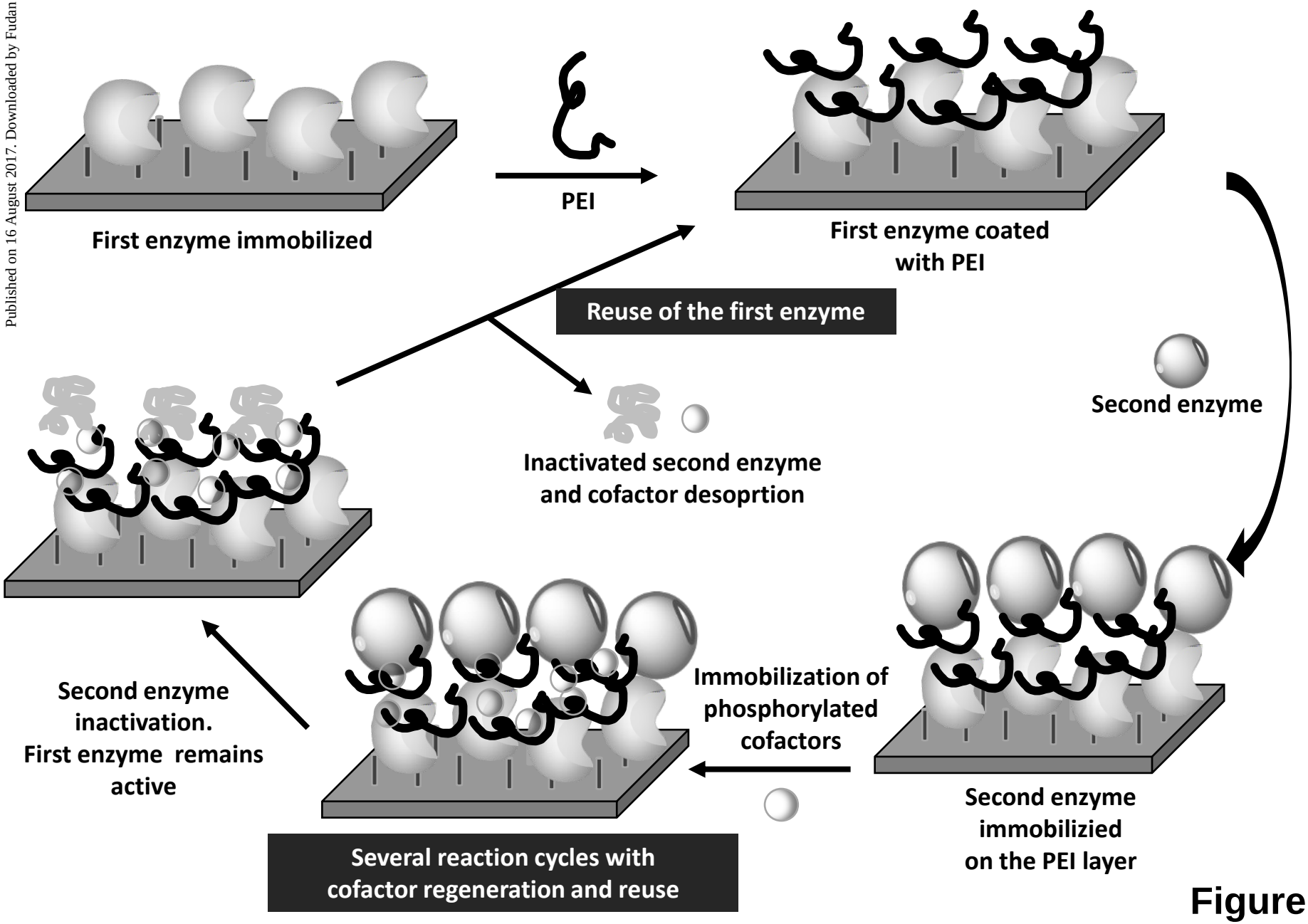
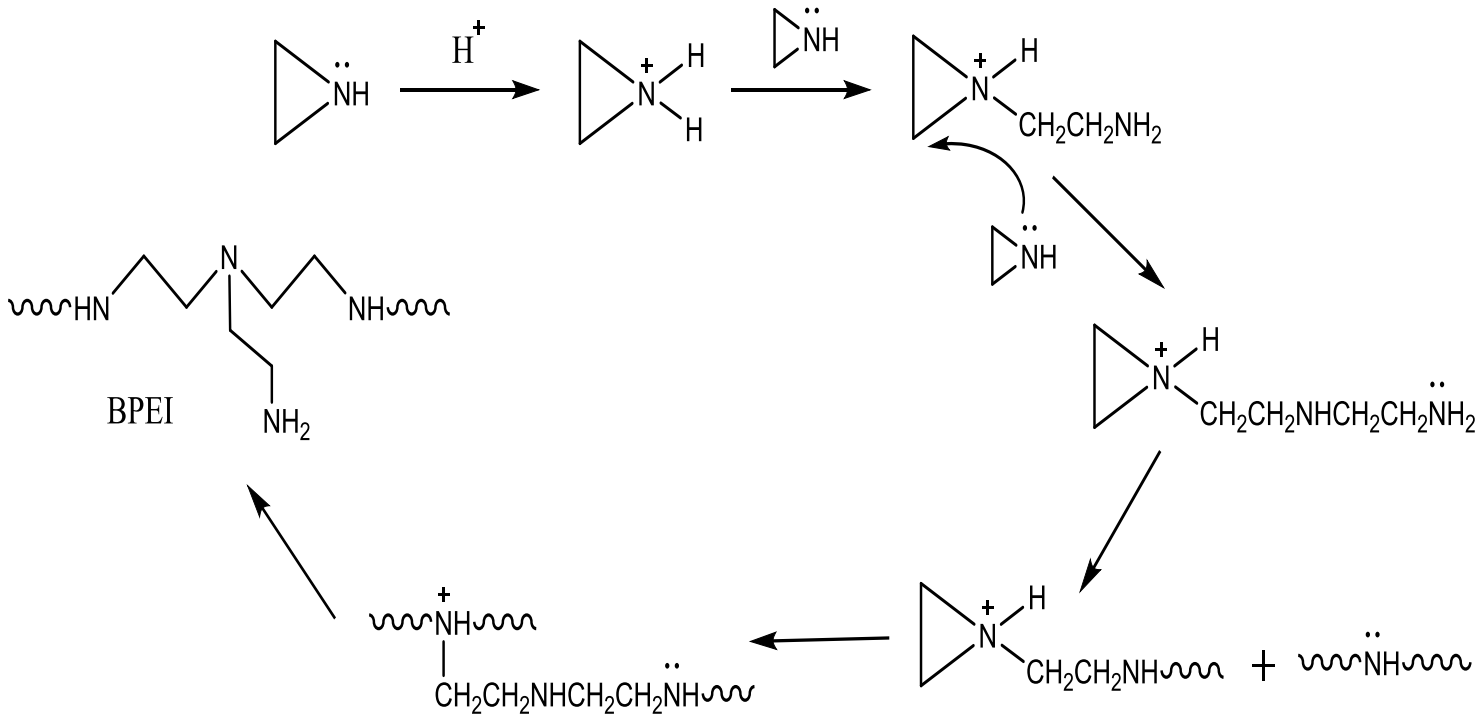
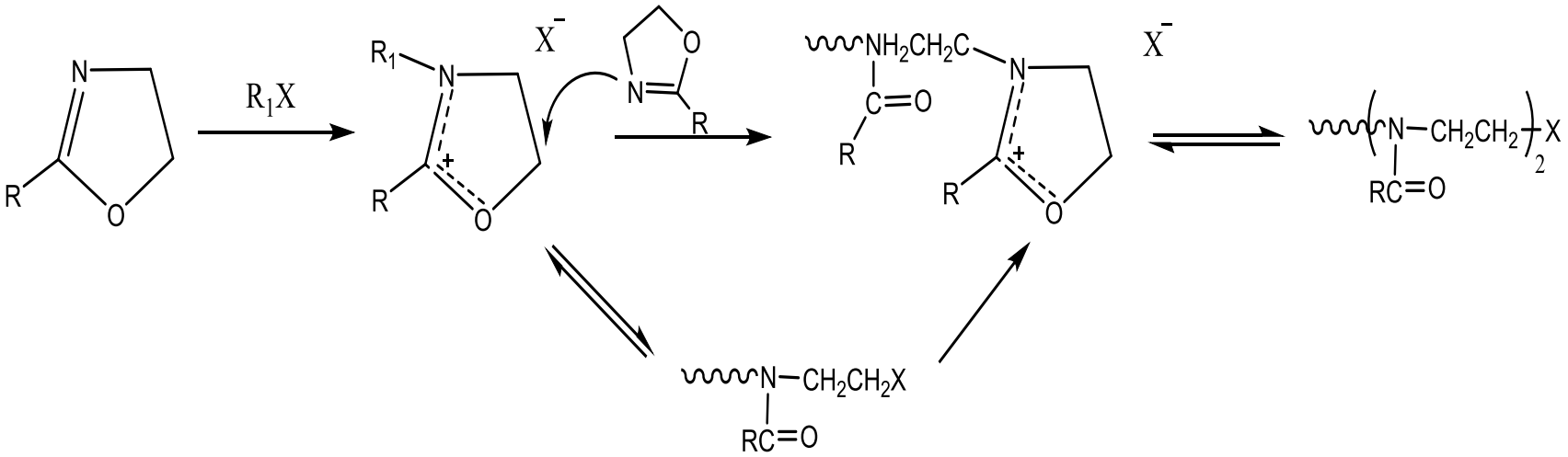


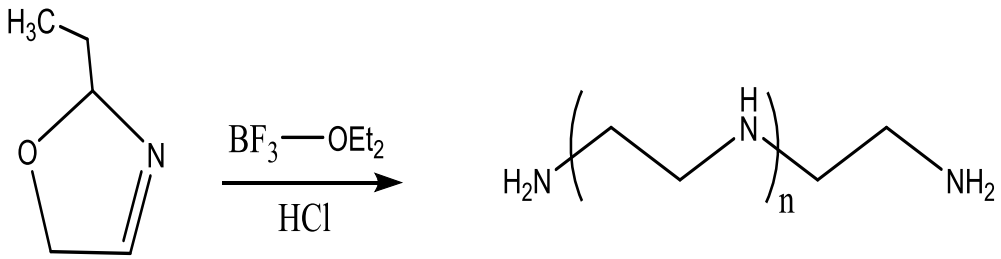
Figure 21



Scheme 1



Scheme 2



Scheme 3